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# Nitrogen Fixation in Bacteria and Higher Plants

With 27 Figures

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# Preface

Our knowledge of the biochemistry and biophysics of dinitrogen fixation has developed rapidly in the 15 years since the first $N_2$-fixing enzyme system was successfully extracted from a bacterium. This period has produced a literature that now describes the $N_2$ fixation reaction and the nitrogenase enzyme itself in sophisticated terms, though a detailed reaction mechanism at the chemical level has not yet emerged. It is the purpose of the present monograph to present an in-depth review, analysis, and integration of this research as is possible with a non-contributed publication and to relate this work to considerations of $N_2$ fixation that reach beyond the confines of the biochemist's laboratory.

The first section is directed as much toward the general science reader as toward the specialist. It covers the agricultural origins of man's interest in $N_2$ fixation and also pertinent areas of taxonomy, physiology, and evolution. Ecological aspects of the subject include a comprehensive evaluation of the nitrogen cycle leading to a substantially greater estimate of the rate of global $N_2$ fixation than previous ones. The treatment is of a survey fashion, in part to provide a general over-view of $N_2$ fixation and in part to provide context for the biochemistry and biophysics that follow in the second section. This section begins with a brief chronological review of the biochemical and biophysical research, then treats individual areas of interest topically. These center mainly on the nitrogenase enzyme and the reactions it catalyzes, but also deals with enzyme systems and metabolic activity associated with nitrogenase activity.

Biochemical and biophysical research in $N_2$ fixation has been characterized by productive interactions with a variety of other disciplines, ranging from agriculture and ecology to transition metal chemistry. The debt owed by the laboratory scientist in this field to his more agriculturally oriented associates has been paid at least in part by the development of the acetylene reduction assay that has made possible extensive evaluations of $N_2$ fixation in the field and enabled a dramatic expansion in laboratories exploring all phases of $N_2$ fixation research. The unique character of the nitrogenase enzyme and related proteins such as ferredoxin has attracted the interest of enzymologists as well as specialists in such fields as Mössbauer and nuclear magnetic resonance spectroscopy. As intriguing as the enzyme are the reactions it catalyzes. These include unique triple bond reductions, $H_2$ formation, and ATP hydrolysis, and when these electron transfer and hydrolitic reactions are fully understood, they will certainly provide an interesting chapter in the study of enzyme mechanisms and kinetics. Increasingly, the biological scientist and his abiological counterpart track each other in their respective $N_2$ fixation studies, and non-enzymic catalytic systems, some closely mimicking the enzyme reaction, have already been developed. Both the biol-

ogical and abiological work have obvious potential to upgrade and expand agricultural production. In today's world of pending food crises, few research areas are more relevant to human needs. We hope that the current volume will serve to stimulate further work at all levels of $N_2$ fixation research.

Wilmington, Spring 1975

RICHARD C.BURNS · RALPH W.F.HARDY

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# Part I. The Diazotrophs*

* The term diazotroph is introduced here and defined as any $N_2$-fixing organism or symbiotic association.

# Recognition

## I. Historical Roots*

### A. The Agricultural Imperative

Through most of the world legumes have played a major role in the history of man's efforts to produce food. Nutritional merit alone would probably have earned these plants priority consideration, but what must have assured them the greatest respect among farmers was their unique ability to enrich the soil. The prevalence of various pulse seeds in archeological diggings attests to the early origins of legume culture, though one may only guess when man began to realize that these plants could maintain the fertility of his fields. However, it is not likely that a cause-effect relationship of such economic significance would remain unnoticed for long in the early empirical approach to agriculture. The soil enriching quality of legumes was clearly appreciated by the early Romans, who developed the concept of crop rotation in which legumes figured prominently. This practice persists today as a major instrument in soil management throughout the world. In developed countries the advent of cheap nitrogen fertilizer has currently lessened the importance of legumes, but increasing concern over possible contribution of fertilizer nitrogen to water pollution has stimulated a renewed interest in extending biological $N_2$ fixation.

The fact that legumes "did better" than other plants was not lost on the 19th century experimentalists who attempted to construct a scientific rationale for plant nutrition. For example, Sir Humphrey DAVY observed in his book Agricultural Chemistry in 1813 that "peas and beans in all instances seem well adapted to prepare the ground for wheat.....it seems that the azote which forms a constituent part of (their) matter is derived from the atmosphere." This knowledge exerted a continuing pressure which motivated considerable theorizing and experimentation, and it was the need to explain this unique property of the legumes that led finally to the recognition of dinitrogen fixation as a biological process.

### B. Early Experimentation

In the early history of dinitrogen fixation research the work of HELLRIEGEL and WILFARTH (303) in the 1880's was unquestionably the decisive single contribution. By means of a relatively straightforward series of nutritional experiments on some common agricultural plants,

* Informative accounts of the historical aspects of $N_2$ fixation and the early research developments are found in books by FRED, BALDWIN and McCOY (238) and McKEE (460) and in articles by WILSON (714, 715, 716, 718) and AULIE (25). The factual aspects of the brief history reported here have been distilled largely from these sources.

as in Table 1-1, the two German experimentalists showed unequivocally that:

1. legumes were different from other plants; specifically, they could utilize atmospheric $N_2$,
2. this utilization, or fixation, depended on the active participation of certain microorganisms in a legume-microorganism symbiosis, and
3. the root nodules were the active centers of $N_2$ fixation.

Table 1-1. Typical data of HELLRIEGEL and WILFARTH (303), showing superiority of legumes at low soil nitrogen levels

| Pot No. | Initial Nitrogen in calcium nitrate | Oats Average weight of grain and straw | Peas Average weight of seed and vines |
|---|---|---|---|
| | gm. | gm. | gm. |
| 1 | None | 0.390 | 4.380 |
| 2 | 0.056 | 5.876 | 4.128 |
| 3 | 0.112 | 10.961 | 9.132 |
| 4 | 0.168 | 15.997 | ----- |
| 5 | 0.224 | 21.357 | 9.725 |
| 6 | 0.336 | 30.175 | 11.352 |

Their experiments accomplished several objectives which had eluded all predecessors. Most significantly, they proved to a skeptical scientific community that biological $N_2$ fixation did in fact exist. Further, they identified the requirements of $N_2$ fixation in legumes and clarified why legumes "did better" than other plants under adverse nutritional conditions. Finally, they explained why earlier experimentation by others had failed. Thus the past was explained and the future course was pointed out. Clearly a fresh page had been turned.

In the 50 to 60 years leading up to this work the question of $N_2$ utilization by plants had passed a tumultuous gestation. It engaged the minds of some of the century's most prominent scientists with the result that a confusing, quarrelsome and equivocal literature on the subject had accumulated. By the time HELLRIEGEL and WILFARTH reported their results, the concept of $N_2$ fixation lay in official disrepute for almost thirty years. However, their data were so convincing that the legitimacy of $N_2$ fixation was almost immediately accepted and the concept never again placed in serious doubt.

Why was the definitive experimentation so long in coming? Certainly credit must be given to the authors, WILFARTH and particularly HELLRIEGEL. Yet these men were not of the scientific caliber of LIEBIG or numerous others who had considered but failed to solve the question. Part of the answer is found in the general status of science in the 19th century and in a consideration of $N_2$ fixation within this context. The success of the German workers rested on crucial inputs from such varied disciplines as agronomy, plant physiology, microbiology and chemistry. Their experimentation was truly a multidisciplinary accomplishment which could hardly have occurred before these different fields had reached an appropriate maturity.

The possibility that plants might be able to utilize $N_2$ began to receive consideration soon after RUTHERFORD discovered in 1772 that $N_2$ was a major atmospheric component. By the end of the eighteenth cen-

tury the major components of the atmosphere had been correctly identified. The remarkable involvement of $O_2$ and $CO_2$ in life processes was rapidly established by INGEN-HOUSZ, SENEBIER, SAUSSURE and PRIESTLEY, whose findings led to the concept of an aerial nutrition for plants. Dinitrogen appears to have been loosely included in this concept, its participation assumed by PRIESTLEY, INGEN-HOUSZ and DAVY. Though this assumption lacked experimental support, it must have appeared quite logical; the ubiquity of nitrogen in living matter was becoming well established, and if the carbon needs of life could be met with the 0.03% $CO_2$ present in the atmosphere, the massive concentration of $N_2$ in the atmosphere could reasonably be expected to satisfy nitrogen needs.

The first recorded experimental work appears to be tests conducted around 1806 by SAUSSURE. This work was done with potted plants and formed part of SAUSSURE's pioneering efforts to extend chemical techniques and quantitation into biology; the tests indicated that the soil, not the atmosphere, was the source of plant nitrogen. Others who considered the question of aerial dinitrogen assimilation included GAY-LUSSAC and HERMSTÄDT, but none of these early experimentalists developed a detailed or extended investigation of the question.

A more enduring commitment to this problem was initiated by Jean Baptiste BOUSSINGAULT in France. BOUSSINGAULT was among the first to pursue the quantitative chemistry of field crops, and the question of nitrogen nutrition occupied a major place in his research. Through his own early experimentation and from more preliminary analyses by VAUQUELIN and FOURCROY, BOUSSINGAULT was aware that the superiority of legumes over cereals was due to the high level of nitrogen in the legumes. Based on analyses of crop rotations which included clover and which extended for 4 to 5 years on hectare size plots, he concluded that the atmosphere supplied nitrogen to the plants, and he announced this conclusion to the French Academy in 1838 after obtaining supporting data from pot experiments with clover and wheat. His early experimentation showed that nitrogen was the key component of added fertilizers and that legumes enriched the soil by introducing nitrogen from the air; the experiments did not define the nature of the nitrogen as specifically $N_2$, airborne organic particulates or gaseous ammonia.

The identity of the functional atmospheric species appeared to be resolved in favor of ammonia as a result of the strong support for this source provided by LIEBIG in his influential "Chemistry and Its Application to Agriculture and Physiology", published in 1840. Ammonia had been recognized as a possible source by almost all who had considered the question of plant nitrogen nutrition, and though LIEBIG offered no new data, his persuasive arguemnt led now to general acceptance of the ammonia theory. Less well received, however, was LIEBIG's mineral theory of plant nutrition in which he dismissed nitrogen as a trivial component of fertilizers and acknowledged minerals to be the only effective components. Though this concept denied BOUSSINGAULTS's work and conclusions, BOUSSINGAULT did not take issue with LIEBIG, and it was rather LAWES and GILBERT at the Rothamsted Experiment Station who challenged this theory. Extensive field experiments involving crop rotation had convinced the Rothamsted workers that BOUSSINGAULT's contributions were valid and the mineral theory incorrect; these views became generally accepted in the 1850's following more than 10 years of heated debate in the literature.

Meanwhile BOUSSINGAULT had begun a second series of experiments on the source of nitrogen. These appear to have been prompted by the work of Georges VILLE, who in 1850 began to report considerable evidence in support of $N_2$ fixation by potted plants. Both workers reportedly grew their plants in closed systems from which ammonia was excluded, and most significantly both used calcined soil. BOUSSINGAULT observed consistent negative results and concluded that plants could not use $N_2$. In contrast VILLE continued to report substantial $N_2$ fixation; however, his results could not be verified by LAWES, GILBERT and PUGH at Rothamsted in a series of experiments in 1857, 1858, and 1861, even using apparatus sent to them by VILLE. In retrospect it is difficult to rationalize the positive results of VILLE with his use of calcined soil. This practice of exhaustively roasting the soil before use, which clearly doomed their best experimental efforts to failure, was apparently never considered to be an important consideration by any of the early experimenters.

In the course of his work during this period BOUSSINGAULT undertook extensive analyses to determine whether the atmosphere contained sufficient ammonia to sustain plant requirements. He concluded that it did not, and his results were verified in London in 1856 by WAY, who extended the analysis to include atmospheric nitrate and showed that even the combined sources were inadequate. Attempts to implicate atmospheric sources of nitrogen in plant nutrition effectively ceased following publication of the Rothamsted report of 1861, and were not resumed for almost 25 years. With the atmospheric sources apparently discredited and with the mineral theory now in general disrepute, attention was logically turned to the soil as the most likely source of nitrogen during this period.

Though little direct experimentation on $N_2$ fixation was undertaken during this time, the information which would contribute to a full understanding of $N_2$ fixation was in fact being accumulated through other apparently unrelated findings. LACHMANN, in investigations of the legume root nodules which were so consistently overlooked by the chemists, established the presence therein of "vibro-like" bodies in 1858, and WORONIN in 1866-67 spoke of these as "bacterial-like". In 1862 JODIN reported $N_2$ fixation by microorganisms based on growth in nitrogen-free media and on an observed uptake of $N_2$, but the work was not pursued after an unfavorable reception by a French Academy Committee of Examination; BOUSSINGAULT's studies on soil nitrogen during the period of 1860-1876 led to the clarification of the major chemical features of nitrification (p. 40), and showed LIEBIG's early theory of this phenomenon to be essentially correct. In 1877 SCHLOESING and MÜNTZ established the role of bacteria in nitrification, and initiated the novel concept of participation by micro-organisms in soil chemistry. That they might also play a role in reactions with $N_2$ was implicit in JODIN's early results (above), and was further indicated by BERTHELOT who observed an increase in soil nitrogen that could be prevented if the soil were first heated at 100°C.

Persisting throughout this period, as before, was the certain knowledge among agriculturists that under field conditions legumes "did better" and possessed definite soil-enriching properties. LAWES and GILBERT in fact pointedly observed that legumes in the field in contrast to the legumes grown under their rigidly controlled experimental conditions contained unaccounted for nitrogen. Others, including SCHULTZ-LIPITZ in Germany and ATWATER in the U.S.A., made similar observations and pointed out the inadequacy of current dogma to account for their findings. It was in this atmosphere of extreme un-

certainty about the existence of $N_2$ fixation that Hermann HELLRIEGEL, independently at first and then with WILLFARTH, conducted the experiments which yielded the long sought solution. Unlike all predecessors they examined the significance of root nodules in the nitrogen nutrition of plants, and this of course proved to be the key piece of information leading to an explanation of $N_2$ fixation by legumes.

## II. Detection of Diazotrophs and Nitrogenase

Other scientists were quick to build on the foundation laid by HELLRIEGEL and WILLFARTH, and a period of important productivity and discovery followed their work. In 1888 BEIJERINCK isolated the nodule bacterium and named it *Rhizobium radicicola*, subsequently changed to *R. leguminosarum*. NOBBE suggested $N_2$ fixation by a nodulated non-legume, *Elaeagnus angustifolia*, in 1892, and $N_2$ fixation by free-living microorganisms was established by WINOGRADSKY's demonstration in 1893 that *Clostridium pasteurianum* could fix $N_2$. The momentum that appeared to be building up in $N_2$ fixation research, however, was not sustained. An increasing flow of reports describing $N_2$ fixation by various free-living microorganisms began to appear in the literature, but the lack of a sufficiently sensitive assay, and often the lack of sufficiently pure cultures, made $N_2$ fixation and the $N_2$-fixing agents difficult to establish with certainty. It became lamentably clear, as WILSON pointed out, that "Nature failed to provide a bright-hued signal comparable to chlorophyll to mark the nitrogen fixers" (713). More significantly, existing technology was unable to overcome this oversight of Nature, and interest in $N_2$ fixation research declined as investigators turned to fields that could be more readily exploited.

Ostensibly, the qualification test for a candidate diazotroph should be simple and direct: either an organism would grow in the presence of $N_2$ and absence of fixed nitrogen, or it would not. In practice, however, such a direct approach seldom met with success. The difficulties encountered proved to be both considerable and at least partly unexpected. The extraordinary ability of numerous microorganisms to scavenge fixed nitrogen was not well appreciated, and the resultant growth caused considerable problems; these difficulties were frequently compounded by the impure nature of available reagents or the presence of atmospheric ammonia in miniscule, but decisive amounts. Furthermore, it is now apparent that $N_2$ fixation is not characterized by a high degree of taxonomic continuity, but rather by considerable variation in specificity with respect to family, genus and even species, and these inconsistencies precluded an effective phylogenetic approach to the identity of the diazotrophs.

The general ignorance of the conditions which best supported - or, in fact, were absolutely required for - $N_2$ fixation imposed additional obstacles; for example, the repressive effect of fixed nitrogen on nitrogenase was not understood, nor was the need for anaerobic conditions by facultative diazotrophs known. Even today the inability of a particular isolate to fix $N_2$ in the laboratory may simply reflect the investigator's inability to cater to what may be very exacting requirements, as suggested in recent reports on the importance of reduced $pO_2$ (p. 102) for certain aerobic diazotrophs and the apparent beneficial effect of certain non-$N_2$-fixing organisms on the fixation rates of known diazotrophs. A pertinent case is the recent demonstration of $N_2$ fixation in *Aphinosomen flos-aquae* (661): evidence for fixation was obtained using $C_2H_2$ reduction and $^{15}N$ analyses,

whereas earlier $^{15}N$ assays indicated no fixation (711). The failure to provide the most favorable conditions for $N_2$ fixation for a given agent is probably also at the heart of the apparently great variability in the facility with which known diazotrohs actually fix $N_2$, though much intrinsic variation also no doubt exists. Whatever the cause, certain organisms, e.g., *Azotobacter* and *Clostridium*, are well known to be high activity fixers, while numerous others are not. An interesting case is that of the bacterium *Derxia gummosa*, reported variously as fixing $N_2$ with "unusually high efficiency" and subsequently as being a "typical awkward $N_2$ fixer" (308, 562).

An additional difficulty associated with early investigations was the lack of a suitable analytical method for much of this work. In fact, little real progress was made until analytical methodology advanced. The Kjeldahl method for determination of nitrogen content was introduced in 1883, just prior to the experimentation which established that $N_2$ fixation did in fact exist. This method was better than the Dumas procedure for most applications, and it formed the basis for determination of $N_2$ fixation for the next fifty years. While unquestionably a good quantitative method, the Kjeldahl nitrogen determination method had serious limitations for detection of $N_2$ fixation. The amount of nitrogen fixed, as determined by the Kjeldahl method, is measured as the difference between total nitrogen found in the test material before and after growth on $N_2$; since this frequently meant accepting a very small difference between two large values as evidence for $N_2$ fixation, small variations in sampling or techniques easily led to erroneous conclusions. It is not surprising then that as late as 1948 only the *Rhizobium*-legume associations (obligatory symbioses), *Clostridium* and *Azotobacter* (free-living bacteria) and *Nostoc* (a blue-green alga) were critically accepted as agents of $N_2$ fixation, though many other candidates were promoted with variable merit and enthusiasm.

As research in $N_2$ fixation and analytical techniques advanced, a variety of assay methods for $N_2$ fixation were developed. These are listed in Table 1-2, which also includes the applicability and principle of each method. The most commonly used are discussed below.

Table 1-2. $N_2$ fixation assays

| Assay method and principle | $N_2$-fixing system | | |
|---|---|---|---|
| | Nitrogenase *in vitro* | Organisms in culture | Field and natural systems |
| Growth and morphology | | | |
| Increase of biomass or optical density in N-free medium | | ++ | ++ |
| Heterocyst detection (algae) by observation | | ++ | ++ |
| Nitrogen-based methods | | | |
| Increase in N content after growth on $N_2$ -Kjeldahl or Dumas | | ++ | ++ |
| $^{15}N$ or $^{13}N$ enrichment in $NH_4^+$, cell components after exposure to $^{15}N_2$ or $^{13}N_2$ | | | |
| -mass spectrometry ($^{15}N$) | ++ | ++ | ++ |
| -optical mass emission ($^{15}N$) | | + | + |
| -radioactive counting ($^{13}N$) | + | + | |

Table 1-2 (continued)

| Assay method and principle | $N_2$-fixing system | | |
|---|---|---|---|
| | Nitrogenase *in vitro* | Organisms in culture | Field and natural systems |
| Decrease in $N_2$:Ar ratio following incubation | | | |
| -mass spectrometry | | + | |
| Ammonia formation in extracts following incubation under $N_2$ | | | |
| -titrimetry | ++ | | |
| -colorimetry | ++ | | |
| $N_2$, $H_2$ uptake by extracts | | | |
| -manometry | + | | |
| Methods based on ability of nitrogenase to reduce substrates other than $N_2$ | | | |
| $C_2H_4$ reduction to $C_2H_4$ | | | |
| -gas chromatography | ++ | ++ | ++ |
| -colorimetry | | | + |
| Nitrile, isonitrile reduction | | | |
| -gas chromatography | + | + | |
| $H_3O^+$ reduction to $H_2$ | | | |
| -manometry | ++ | | |
| -gas chromatography | + | | |
| -mass spectrometry | + | | |
| Method based on utilization of dithionite | | | |
| -u.v. spectrophotometry | + | | |

\+ Indicates at least one report;
++ indicates frequent use.

## A. $^{15}N_2$ Method

The first major technical advance in $N_2$ fixation detection was the application of the stable isotope $^{15}N$ to $N_2$ fixation studies. The methodology and applications of $^{15}N$ were devised soon after techniques for preparation of $N_2$ enriched in $^{15}N$ were developed in the 1930's. BURRIS and MILLER (113) demonstrated that organisms could not incorporate $^{15}N_2$ by exchange and thereby established that assimilation of $^{15}N_2$ was definitive proof for $N_2$ fixation, a criterion which still holds. It has subsequently been demonstrated with *Azotobacter* that there is no isotope effect in the rate of fixation of $^{14}N^{15}N$ vs. $^{14}N^{14}N$ (312).

The method provided a sensitivity about 1000-fold greater than that of KJELDAHL's method and formed the basis for much of the biochemical and bacteriological work done on $N_2$ fixation during the 1940's and 1950's. The basic technique, described by BURRIS and WILSON (114), consists of 1. incubation of the test material in the presence of $^{15}N$-enriched $^{15}N_2$, 2. conversion of all N in the sample to ammonia by Kjeldahl digestion, 3. isolation of the ammonia by distillation, 4. oxidation of the ammonia to $N_2$ and 5. mass analysis of the $N_2$ in a mass spectrometer, preferably an isotope ratio model for maximum sensitivity. Any $^{15}N_2$ incorporated by the test material is detected as an increase in the abundance of mass 15 above the background value of 0.364% due to the natural abundance of $^{15}N$. This increase is ex-

pressed as atom % $^{15}N$ excess. Taking the sensitivity of the method into consideration, BURRIS and WILSON (114) recommended that samples giving an atom % $^{15}N$ excess value of at least 0.015 could conservatively be accepted as positive evidence for $N_2$ fixation. With this technique, $N_2$ fixation by the accepted diazotrophs was confirmed, and the validity of numerous questionable claimants was tested during the 1940's (e.g. Ref. 111); however, no new names were added to the list until 1949 when KAMEN and GEST (360) demonstrated $^{15}N_2$ incorporation by *Rhodospirillum rubrum*. They had been led to suspect a $N_2$-fixing capacity in this organism from the observation that $N_2$, as well as $NH_4^+$, inhibited photo-evolution of $H_2$, a particularly suspicious effect in view of the emphasis placed on the hydrogenase-nitrogenase relationship during that period (p. 72). The $^{15}N_2$ method was used extensively through the following two decades in the identification of diazotrophs and in studies with intact $N_2$-fixing agents. Kinetic studies of $^{15}N_2$ incorporation by intact *Azotobacter* and *Clostridia* (108) supported the concept that $NH_3$ was the "key intermediate" in $N_2$ fixation (the first detectable nitrogen compound after $N_2$; Ref. 717). During this period the $^{15}N_2$ method was also used in the continuing efforts to obtain cell-free bacterial extracts capable of fixing $N_2$, and the successful accomplishment of this important goal (p. 65) was reported in terms of $^{15}N_2$ incorporation. In recent years the new technique of optical emission spectroscopy has been used for $^{15}N$-enrichment analysis (471, 554), and the method has been applied to the study of $N_2$ fixation by *Hippophaë* (4). Though less accurate than mass spectrometry, the method requires only very minute sample amounts.

## B. Ammonia and Manometric Methods

The $N_2$-fixing extracts of *Clostridium pasteurianum* prepared according to the procedure of CARNAHAN et al. (120) proved to be the means by which $N_2$ fixation research entered its more purely biochemical and enzymological phase. It was rapidly apparent that these extracts fixed $N_2$ into $NH_4^+$, and also that the levels of $NH_4^+$ were sufficient to permit analysis of $N_2$ fixation by colorimetry or titration. In practice $NH_4^+$ was first isolated by alkaline distillation using the Conway technique (486) or a modification of it (91, 484), and it was then determined quantitatively by the Nessler color reaction or by acid titration. Manometric methods for measuring nitrogenase activity with extracts were also developed. One procedure is based on the uptake of $N_2$ and $H_2$ and is applicable for acellular nitrogenase reactions in which $H_2$ is the electron donor (486); product ammonia does not influence the manometry, since it exists as $NH_4^+$. A second procedure takes advantage of the ability of nitrogenase to evolve $H_2$ in the absence of added reducible substrates (p. 121), which permits facile quantitation from the resulting increase in pressure. The ammonia and manometric methods increase in pressure. The ammonia and manometric methods were much simpler and more rapid than the $^{15}N_2$ analysis. They have been used extensively in cell-free work, though they lack the sensitivity of the $^{15}N$ or acetylene methods and cannot be used for analysis of $N_2$ fixation by whole cells.

## C. Acetylene Reduction Method

The discovery that nitrogenase could reduce compounds other than $N_2$ led to the most recent major analytical advance in $N_2$ fixation research. In 1966, after $C_2H_2$ was shown to inhibit $N_2$ reduction (171, 598), DILWORTH (171) discovered that nitrogenase could reduce acetylene to ethylene. The analytical possibilities of this substrate coupled

with gas chromatographic analysis were rapidly recognized by HARDY and KNIGHT (289), and subsequent developmental work showed that acetylene reduction did in fact possess attributes far superior to all previous analytical methods (285). The versatility of the method has made it equally applicable in the field and in the laboratory, and it has resulted in a surge of analytical activity unprecendented in $N_2$ fixation research. Its sensitivity and other advantages have fostered laboratory experimentation which had once been technically unfeasible, but its main impact has been in the analyses of $N_2$-fixing activity in the field under both natural and agronomic conditions. A recent review (274) of the utilization of the acetylene reduction assay revealed world-wide application resulting in over 200 literature reports up through 1971. These include analyses of $N_2$ fixation by isolated nitrogenase and $N_2$-fixing organisms in the laboratory; free-living agents in crop lands, grasslands, rice paddies and marine environments; analyses of legumes, varying from limited investigations to comprehensive profiles of $N_2$ fixation by legume crops through complete growth cycles, and analyses of $N_2$ fixation by non-legume symbionts. These analyses comprise the initial data for the mapping of global $N_2$ fixing activity which is essential to understanding and evaluating the role of $N_2$ fixation in the biosphere.

The analyses are conducted by incubating the test material in a gas-tight container which contains a partial pressure of acetylene. At appropriate times samples of the atmosphere may be withdrawn by syringe for immediate or eventual analysis by $H_2$-flame after gas chromatography. The amount of $C_2H_4$ detected is correlated with the intensity of nitrogenase activity in the sample.

Because of the increasing use of the $C_2H_2$ reduction assay for evaluation of $N_2$ fixation, it has been proposed that the bracketed, italicized acetylene formula, $[C_2H_2]$ be used to designate $N_2$ fixation values which are derived from $C_2H_2$ reduction assays (274). This convention will be used in the present volume, as in $N_2[C_2H_2]$-fixing activity and nitrogenase $[C_2H_2]$ activity.

Extensive testing of the $C_2H_2$ reduction assay (285) showed that the biological characteristics of $C_2H_2$ reduction paralleled those associated with $N_2$ reduction, and that the reaction was catalyzed by representatives of all major classes of diazotrophs, as well as by cell-free preparations. In quantitative comparison to $N_2$ reduction, the reduction of 3 to 4 moles of $C_2H_2$ to $C_2H_4$ was equivalent to the reduction of one mole of $N_2$. Kinetic analyses showed that $N_2$-fixing systems were readily saturated by low levels of $C_2H_2$ with $K_m$ values in the range of 0.002 to 0.009 atm. Ethylene was the only product, and no evidence has been obtained for its further metabolism.

In addition to its biological attributes, the assay provides distinct advantages of a strictly analytical nature, including:

1. Sensitivity: it is about $10^3$ times more sensitive than $^{15}N_2$.
2. Facility: because both $C_2H_2$ and $C_2H_4$ are gases, the application of substrate $C_2H_2$ and recovery of product $C_2H_4$ require only simple gas handling techniques; the gas chromatograph and incubation equipment are likewise simple to use.
3. Internal standard: $C_2H_2$ is a natural internal standard for the assay and it is measured readily in conjunction with the product $C_2H_4$.
4. Specificity: product $C_2H_4$ is readily separated and quantitated on the gas chromatograph.

5. Rapidity: about 80 incubations, and about 160 gas chromatographic analyses per man-day are feasible.
6. Product stability: the gas samples containing product $C_2H_4$ can be stored indefinitely in gas-tight containers prior to chromatographic analyses.
7. Economy and mobility: all apparatus used is relatively inexpensive and rugged enough for use in all-terrain-type mobile units.
8. Sample conservation: the test material need not be sacrificed for analysis and normally can be repeatedly sampled because each analysis requires only a small fraction of the incubation atmosphere.

The disadvantages of the method include the explosive nature of $C_2H_2$ and the indirect nature of the reaction in the sense that $C_2H_2$ is not the physiological substrate. In most studies the correlation between $C_2H_2$ reduced and $N_2$ reduced indicates that the reduction of 3 to 4 moles $C_2H_2$ reflects nitrogenase activity capable of reducing 1 mole $N_2$. This relationship is consistent with the amount of reductant needed, since $C_2H_2$ reduction requires $2e^-$ and $N_2$ reduction requires $6e^-$; however, variability in the conversion factor has been observed (45, 85, 582), and the most reliable data for given conditions are obtained by establishing a factor for each particular variation in experimental procedure. Finally, the possibility of $C_2H_2$ reduction to $C_2H_4$ by means other than nitrogenase catalysis must be considered, as must the possibility of $C_2H_4$ consumption by unknown sample components. To date neither of these possibilities has been found to compromise analytical results.

An application of the acetylene reduction assay beyond the area of dinitrogen fixation was developed by STEWART, FITZGERALD and BURRIS (662) who have used the method to evaluate phosphorous availibility in aquatic environments. In their procedure phosphorous-starved *Anabaena* are incubated in the water sample and reduce $C_2H_2$ at a rate which is proportional to the amount of phosphorous availabile in the sample.

## D. Other Methods

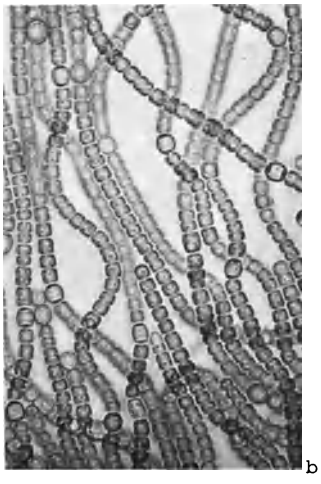
A recently reported procedure which promises to be of considerable use in kinetic studies of purified nitrogenase preparations is based on the decrease in dithionite absorbance at 315 nm as this substrate is consumed (432). Like the manometric methods this assay permits continuous monitoring of reaction rate, but in addition provides a greater sensitivity. In other analytical approaches, reducible substrates other than $N_2$ and $C_2H_2$ have been used for specific applications. Nitriles and isonitriles have been employed for qualitative analysis (241), and acrylonitrile has been particularly useful in comparisons of activity differences attributable to the replacement of Mo by V in *Azotobacter* nitrogenase (100). Evaluation of $H_2$ evolution by gas chromatography has been demonstrated and can be particularly useful for determining $H_2$ in the presence of other gaseous products or substrates (241). The application of the radioactive nitrogen isotope, $^{13}N$, to $N_2$ fixation studies has been used to a limited extent (117, 519), but the restrictions imposed by the short half-life (11 min) of this nuclide have discouraged wide usage in spite of a theoretically high sensitivity. A mass spectrometric method which does not require $^{15}N_2$ involves analysis of changes in the ratio $N_2$:Argon as a result of $N_2$ uptake (223). The method is quite

insensitive and appears to offer no advantages over other techniques. A final method, applicable only to blue-green algae, is the detection of heterocysts by light microscopic examination (p. 23). The presence of these specialized cells suggests that the alga has the capacity to fix $N_2$, but no quantitative significance is indicated.

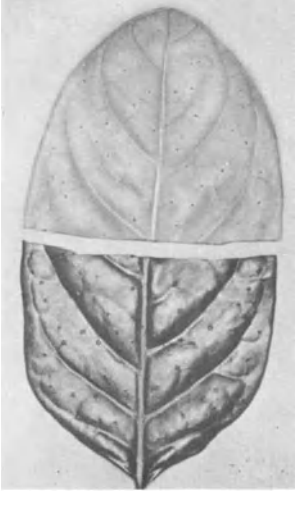
# Description and Classification of Diazotrophs

In comparative biology it is normally observed that organisms possessing a common function also tend to have certain distinguishing physiological, metabolic, structural and genetic characteristics. The organisms which can use $N_2$ to meet their nitrogen requirement, however, appear to violate this rule: except for the ability to fix $N_2$, these organisms, the diazotrophs, have little else in common. They comprise

a

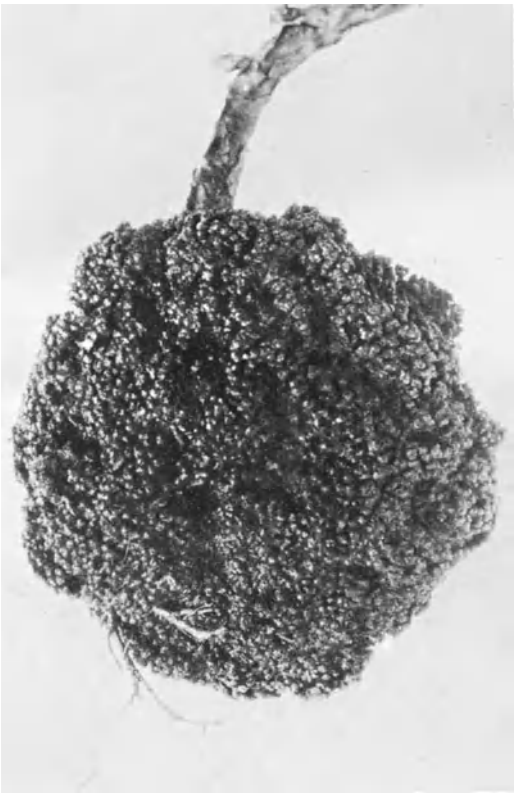


b

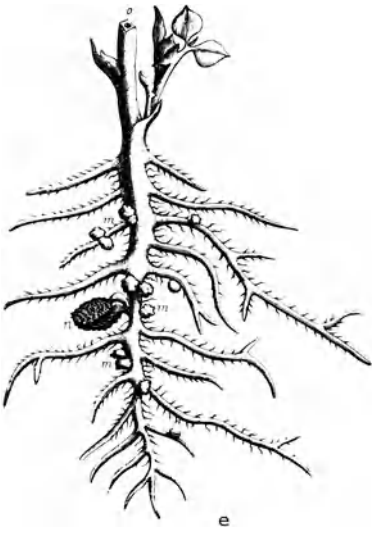


c

Fig. 2-1. a to c



d



e

Fig. 2-1. d to e

Fig. 2-1. a to e. Representative diazotrophs. (a) Free-living bacterium, *Azotobacter vinelandii* (electron micrograph), courtesy J.T. STASNY. (b) Filamentous blue-green alga, *Anabaena variabilis* (X 450), showing heterocysts (enlarged cells), courtesy W.E. SCOTT. (c) Leaf nodule symbiosis, *Psychotria-Klebsiella* (X 2), courtesy W.S. SILVER. (d) Non-legume angiosperm root nodule symbiosis, *Eleagnus* (Ref. 238); (e) Legume root nodule symbiosis, *Vicia faba-Rhizobium*, as depicted in wood-cut by MALPIGHI, 1679 (Ref. 238)

an incongruous mixture made up primarily of certain bacteria and algae, but also encompassing, in a unique participation, certain higher plants. However, it appears increasingly evident that the enzyme nitrogenase occurs naturally only in prokaryotic cells. Some genetic bridges and barriers do appear to exist among them, but often with little obvious logic. The inclusion of aerobic, facultative, anaerobic and photosynthetic organisms among the diazotrophs attests to their physiological and metabolic diversity. Representatives of major classes of diazotrophs are shown in Fig. 2-1.

The diazotrophs vary in complexity from the primitive bacterium, *Clostridium*, through the physiologically and genetically intricate legume root nodule symbioses. Between these extremes $N_2$-fixing organisms are found at most levels of development among the bacteria, sporadically through the blue-green algae, and also in symbiotic as-

sociations with representatives of the main taxa of higher plants, many of which form distinct physiological structures to accommodate the $N_2$-fixing organism. Examination of the numerous interorganismal relationships associated with $N_2$ fixation reveals a gradation in the degree of intimacy between partners. This is minimal in the loose associations between free-living microorganisms, but intensifies as higher plant forms participate and culminates in the highly complex root nodule system (Fig. 2-2). The most obvious consequence of these symbioses is a quantitative effect on the amount of $N_2$ fixed; however, in the most intimate symbioses the overriding consideration is qualitative. In these, establishment of symbiosis influences not only the amount of $N_2$ that will be fixed, but it also determines whether a competent $N_2$-fixing enzyme system will even be formed in the first place. These symbioses bring together two species, neither of which can fix $N_2$ independently, and which, through mechanisms not yet understood, create a $N_2$-fixing system. The inability of either partner to fix $N_2$ independently is the qualifying characteristic which distinguishes these obligatory symbioses from the associative type. In the latter, one of the partners is a genuine free-living diazotroph in its own right, and it brings this function into the symbiosis. The *Rhizobia* - legume root nodule symbioses are the only definitive examples of obligatory symbiosis. Root-nodule symbioses of the alder type are candidates, but until the endophytes are successfully cultured and examined no definitive assignment of these symbioses can be made. The possibility also exists, of course, that some of the less investigated symbioses may qualify as obligatory symbioses.

In the following discussion the free-living diazotrophs are considered first as independent microorganisms and then within the context of the associations they form with other organisms. The angiosperm root nodule systems are categorized as either legume or non-legume symbioses. The lists of organisms in this section comprise the currently accepted diazotrophs and are complete to the authors' knowledge; nevertheless, they are presented only as an interim summary, since without doubt additions and possibly deletions will be made. The fact that several genera listed here were reported since 1969 attests to the transient nature of such listings. As significant, but less evident, is the omission of species present on earlier lists, but since shown not to fix $N_2$. For example, recent retesting of certain putative $N_2$-fixing organisms (468, 469, 714) has resulted in the removal of molds, yeasts and certain bacteria from the listings, and the genus *Azotomonas*, created specifically to accommodate certain presumed diazotrophs, appears now to contain no qualifying species (538).

Claims for $N_2$ fixation by agents which lie outside generally accepted categories continue to appear in the literature. These require further verification before receiving full accreditation. Included are claims for $N_2$ fixation by species of *Escherichia* (49, 431), *Corynebacterium* and *Nocardia* (406), *Arthrobacter* (640), *Brevibacterium* (407), *Aspergillus* (472) and cellulose-decomposing fungi (514); $N_2$ fixation has also been claimed for bee pupae, silkworm chrysalides and hen eggs (699). Recent reports of $N_2$ fixation by quail embryos (262, 700) have already been refuted (749).

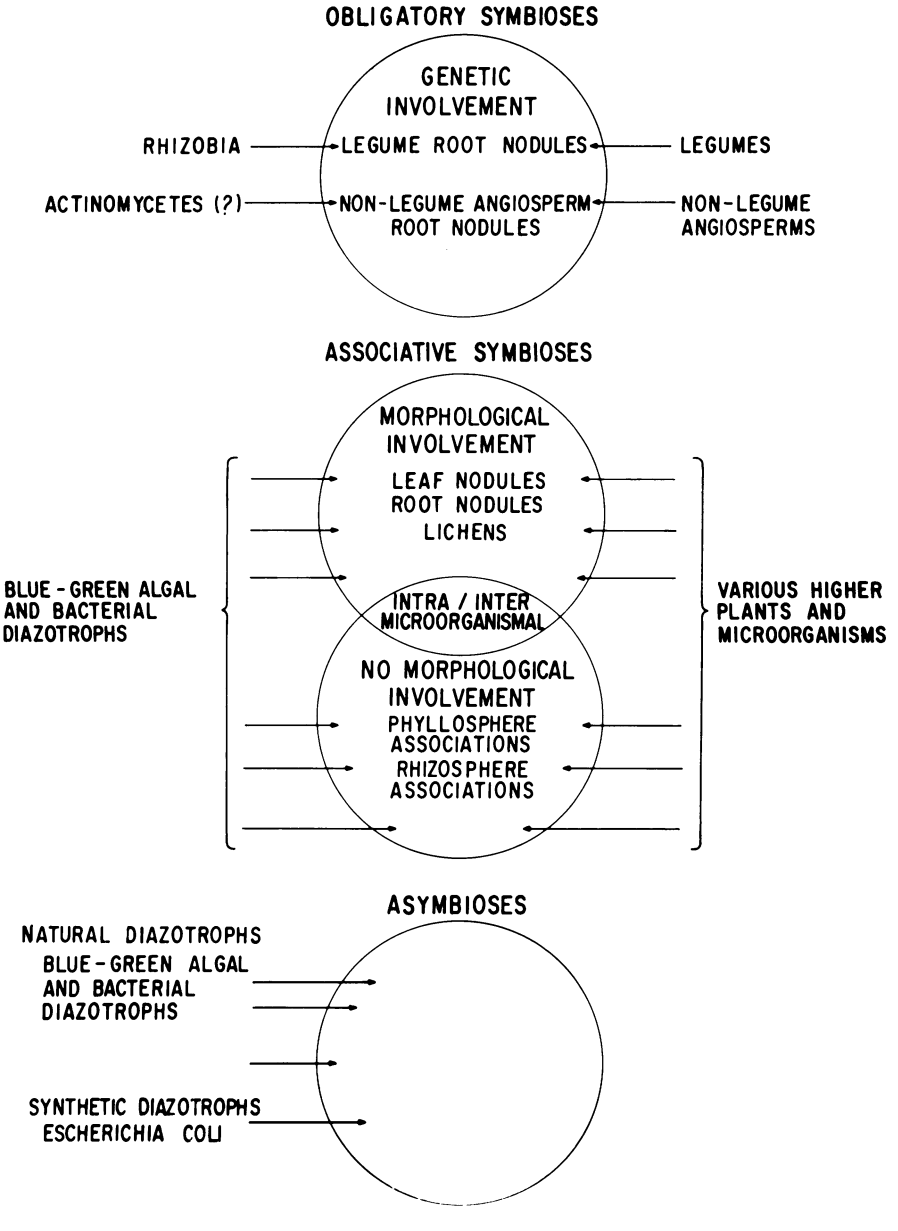



Fig. 2-2. Biological $N_2$-fixing relationships

# I. Free-Living Diazotrophs

## A. Taxonomy and Characteristics

### *1. Bacteria*

Bacterial diazotrophs are indicated in about 26 genera, 11 families and 3 orders of the approximately 200 genera, 47 families and 10 orders of bacteria listed in *Bergey's Manual of Determinative Bacteriology* (54). Included in these 26 genera is the genus *Rhizobium*, whose members have been found to fix $N_2$ only in symbiosis with legumes. If $N_2$ fixation should prove to be a universal trait among the photosynthetic bacteria, the approximately 17 additional photosynthetic genera which have not yet been tested for $N_2$ fixation would be added to the above 26. The list of accredited diazotrophs contains both aquatic and soil species and covers a wide range of metabolic types. Aerobes, anaerobes and facultative species are all represented, though the facultative species fix $N_2$ only anaerobically, and even the aerobes show varying degrees of $O_2$ sensitivity in their nitrogenase activity. The *Azotobacteriaceae* comprise the only family for which the ability to fix $N_2$ is a qualifying characteristic. The consistent occurrence of anaerobic $N_2$ fixation among the photosynthetic bacteria suggests that it may also be universal among the three phototrophic families, but in the remaining taxa, varying degrees of specificity occur as indicated in Table 2-1.

Table 2-1. $N_2$ fixation among the bacteria

| Order | Family | Genus | | |
|---|---|---|---|---|
| | | No. | Incidence of $N_2$ fixation | |
| | | | Well-established | Probable or possible |
| Pseudomonadales | Thiorhodaceae | 14 | *Chromatium*<br>*Ectothiorhodospira* | All untested genera |
| | Athiorhodaceae | 3 | *Rhodopseudomonas*<br>*Rhodospirillum*<br>*Rhodomicrobium* | |
| | Chlorobacteriaceae | 7 | *Chlorobium*<br>*Chloropseudomonas* | |
| | Thiobacteriaceae | 5 | | *Thiobacillus* |
| | Pseudomonadacea | 12 | | *Pseudomonas* |
| | Spirillaceae | 11 | *Desulfovibrio*<br>*Desulfotomaculum* | *Spirillum*<br>*Vibrio* |
| | 4 Other Families | | | |
| Eubacteriales | Azotobacteriaceae | 5 | *Azotobacter*<br>*Beijerinckia*<br>*Derxia*<br>*Azotococcus*<br>*Azomonas* | |
| | Enterobacteriaceae | 10 | *Klebsiella* | |
| | Bacillaceae | 3 | *Bacillus*<br>*Clostridia* | *Methanobacillus* |
| | 9 Other Families | 47 | | |

Table 2-1 (continued)

| Order | Family | Genus | | |
|---|---|---|---|---|
| | | No. | Incidence of $N_2$ fixation | |
| | | | Well-established | Probable or possible |
| Actinomycetales | Mycobacteriaceae | 2 | *Mycobacterium* | |
| | Actinomycetaceae | 2 | | *Nocardia* *Actinomyces* |
| | 2 Other families | 5 | | |
| 7 Other orders | 20 Families | 50 | | |
| 10 Orders | 47 Families | about 200 genera, 18 to 41 containing diazotrophs | | |

Because of the metabolic and physiological diversity (477, 565, 654) among the bacterial diazotrophs, there are few habitats from which bacterial $N_2$ fixation is excluded. Dense populations, however, are generally localized and transient, propagating under favorable conditions and subsequently receding. Factors such as temperature, pH, and moisture influence both the extent of growth and also the types of organisms which will dominate. Aeration is of considerable importance; in most soil samples tested anaerobic $N_2$-fixing activity appears to exceed aerobic activity (85, 123, 583). A critical factor is availability of carbon sources, and soils lacking organic matter normally support only low populations of $N_2$-fixing bacteria (photosynthetic bacterial diazotrophs are mainly aquatic and of little consequence in soil populations). *Azotobacter* and *Clostridium*, long believed to be the only free-living $N_2$-fixing bacteria, are probably the most widespread aerobic and anaerobic genera, respectively, though the ecological ranges of most bacterial diazotrophs are not well defined.

Among the aerobes, *Azotobacter chroococcum* appears to be the most widespread species (473, 476). In very moist soils *Azotococcus* is more prevalent, and in more acidic soils, particularly the tropical laterites, *Beijerinckia* may be the dominant aerobic bacterial diazotroph. DÖBEREINER (184) has described six species of aerobic diazotrophs which appear to be specific for tropical soils. Favored habitats of species of *Azotobacter*, *Beijerinckia* and *Derxia* are the rhizosphere and phylloplane of many higher plants. The organism designated *Spirillum lipoferum* was shown to fix $^{15}N_2$ aerobically (34), but little further work has been reported since its isolation from African soil, and its distribution is unknown. A recent addition to the list of diazotrophs is the aerobe *Thiobacillus ferrooxidans* (444, 617), an organism which thrives under highly acidic conditions and thus has a relatively limited range. The only other presumed free-living aerobic diazotrophs are in the order Actinomycetales and include certain *Mycobacteria (Nocardia?)* and probably *Actinomyces*. The latter are believed to be the endophytes of non-legume angiosperm symbionts, and their distribution is at least as extensive as these; their ability to fix $N_2$ independently has not been shown. The $N_2$-fixing mycobacteria were isolated from forest soils, but the extent of their distribution is not known.

Among the anaerobes the most ubiquitous $N_2$-fixing genus is probably *Clostridium* (476), which has been identified in samples of virtually every soil tested. The *Clostridia* require strictly anaerobic conditions, can tolerate a wide pH range (6.5 ± 2.0) and grow best in the presence

of abundant organic matter. The distribution of other anaerobic diazotrophs is probably much more limited. *Desulfovibrio* and *Desulfotomaculum* are found in marine mud, both fresh and salt water, and in some soils.

Certain *Bacilli* and strains of *Klebsiella pneumoniae* comprise the known facultative non-photosynthetic $N_2$-fixing bacteria. These fix $N_2$ only anaerobically and are widely distributed in both aqueous and terrestrial environments, often associated with decaying animal matter. The photosynthetic bacteria are found chiefly in marine and fresh-water habitats; as with the non-photosynthetic facultative organisms described above, $N_2$ fixation requires anaerobic conditions and, for more than marginal activity, light as well.

In Table 2-1 and in the summary of $N_2$-fixing genera which follows, the taxonomy is based on *Bergey's Manual of Determinative Bacteriology* (54); the numbers of genera and species shown are from this reference, except where indicated, and are adjusted only to include subsequently discovered or reclassified organisms for which $N_2$ fixation has been established.

Order Pseudomonadales: of 10 families, 6 are known to contain diazotrophs.

Suborder Rhodobacteriineae: the photosynthetic bacteria, among which $N_2$ fixation may be a universal trait; $N_2$ fixation occurs only anaerobically, optimally in the light (429).

Family Thiorhodaceae: purple, sulfur photosynthetic bacteria; 14 genera, two known to contain diazotrophs.

1. *Chromatium*. 12 species; $^{15}N_2$ fixation was observed in all species tested, including strain D (24), *C. minutissimum* (166) and unidentified species (740). Cell-free studies have been made with *Chromatium* strain D (24, 721, 724, 740).

2. *Ectothiorhodospira*. $^{15}N_2$ fixation was reported for *E. shaposhnikovii* (747).

Family Athiorhodaceae: purple, non-sulfur, photosynthetic bacteria; 3 genera, all known to contain diazotrophs.

1. *Rhodopseudomonas*. 19 of 20 strains tested from all four species recognized at the time of analysis *(R. paulustra, R. capsulata, R. gelatinosa,* and *R. spheroides)* fixed $^{15}N_2$ (428). Another isolate, indicated to be *R. viridis*, was recently shown to possess $N_2[C_2H_2]$-fixing activity (567).

2. *Rhodospirillum*. 12 species; $^{15}N_2$ fixation was shown for all species tested, including *R. rubrum* Sl (360, 429) and unidentified species (428); cell-free studies have been made with *R. rubrum* Sl (99, 507, 597).

3. *Rhodomicrobium*. $^{15}N_2$ incorporation was shown for the only listed species, *R. vannielii* (428). *Rhodomicrobium* is classified in BERGEY's Manual (54) on the basis of budding, which was thought to distinguish it from *Athiorhodaceae* species; however, budding has since been demonstrated in 3 species of *Athiorhodaceae* (555, 708), invalidating this basis for categorization of *Rhodomicrobium*. A recent attempt to classify it according to % GC content gave equivocal results (618).

Family Chlorobacteriaceae: green, sulfur, photosynthetic bacteria; 7 genera; $^{15}N_2$ fixation shown for two.

1. *Chlorobium*. 2 species; $^{15}N_2$ fixation was shown for *C. thiosulfatophilum* (430), initially erreneously designated as *Chlorobacterium* (415).

2. *Chloropseudomonas*: $^{15}N_2$ and $N_2[C_2H_2]$ fixation were shown for one species, *C. ethylicum* (636, 747).

Family Thiobacteriaceae; 5 genera; $N_2[C_2H_2]$ fixation observed in species of one.

1. *Thiobacillus*. 9 species; $N_2[C_2H_2]$ fixation occurs under aerobic conditions with species of *T. ferrooxidans* (444, 617).

Family Pseudomonadeae: 12 genera; $N_2$ fixation reported under anaerobic and aerobic or microaerophilic conditions (121, 135, 155, 233, 464, 569, 709), but the presumed diazotrophs have not always been isolated or well characterized. Re-examination of those reported to fix makes it appear doubtful whether any pseudomonads fix $N_2$.

1. *Pseudomonas*. An initial report of $N_2$ fixation by *P. azotocolligans* (12) could not be verified (308). Though confirmation was obtained for *P. azotogensis* by both $^{15}N_2$ and $N_2[C_2H_2]$ fixation, anaerobic conditions were required and % GC content indicated that the organism was not a pseudomonad. *P. methanitrificans* gave $N_2[C_2H_2]$ fixation under conditions which may well have been anaerobic or microaerophilic (709); however, reclassification of this organism as a *Vibrio* was indicated from morphological and other tests.

2. *Azotomonas*. 2 species; aerobic $N_2$ fixation was initially reported but could not be verified by recent intensive investigation using $^{15}N_2$ and $^{13}N_2$ (538). Reclassification of existing *Pseudomonas* was suggested from DNA base ratio analysis.

Family Spirillaceae: 11 genera with $N_2$ fixation reported for 3 and possibly one other *(Vibrio)*.

1. *Desulfovibrio*. 7 species; $N_2$ fixation is general among the mesophilic, but absent in the thermophilic species. Anaerobic $^{15}N_2$ and $N_2[C_2H_2]$ fixation was shown for strains of *D. desulfuricans, D. gigas* and *D. vulgaris* (563, 579, 716). Five strains of *D. desulfuricans* fixed $N_2[C_2H_2]$ (563), but single strains of the thermophiles *D. africanus* and *D. salexigens* and of *D. desulfuricans* did not. $N_2$ fixation in this genera, initially reported by LEGALL, SENEZ and PICHINOTY (419), has been particularly difficult to demonstrate consistently (563, 579). $^{15}N_2$ fixation by extracts of an unidentified species was reported (562), but no further work has been described.

2. *Desulfotomaculum*. 3 species; anaerobic $^{15}N_2$ and $N_2[C_2H_2]$ fixation were shown for both strains of *D. ruminis* tested and for the single tested strain of *D. orientis* (563). Attempts to demonstrate nitrogenase in *D. nigrificans* were consistently negative. The genus is described by CAMPBELL and POSTGATE (116).

3. *Spirillum*. 9 species; aerobic $^{15}N_2$ fixation was shown for one tentatively identified as *S. lipoferum* (34), a species suggested to be transitional between *Spirillum* and *Azotobacter* (35); reclassification of the species into the genus *Vibrio* has been suggested by BEIJERINCK (35).

4. *Vibrio*. 34 species; though no species have been implicated specifically as diazotrophs, reclassification could result in the inclusion of numerous examples. WHITTENBURY, PHILLIPS and WILKINSON (709) suggested that the methane oxidizing bacteria be classified as vibrioids. Of 104 strains tested for $N_2[C_2H_2]$ fixation, only one (shown above as *Pseudomonas methanitrificans*) gave a positive response. However, the high $pO_2$ used (0.35 atm)

in the assay may have masked other possible positives. Reclassification of *Spirillum lipoferum* into this genus has been suggested (35).

Order Eubacteriales: the true bacteria; of 13 families, 3 are known to contain diazotrophs, a fourth, the Family Rhizobiaceae, contains the organisms which form the $N_2$-fixing symbioses with legumes, but which do not fix $N_2$ independently.

Family Azotobacteriaceae: the taxonomy is based on DE LEY and PARK (160) and DE LEY (159). 5 genera; all species tested fix $N_2$ aerobically.

1. *Azotobacter*. Species include *A. vinelandii*, *A. chroococcum*, *A. beijerinckii*, *A. miscellum* and *A. paspali* by GC ratio analysis; other reported species include *A. oleovorans* and *A. aromaticum* (135). Much present knowledge of nitrogenase is based on preparations from *A. vinelandii* and *A. chroococcum*; extraction of nitrogenase from other species has not been reported.

2. *Beijerinckia*. Species include *B. indica* (originally *A. indicum*), *B. derxii* and *B. fluminensis*.

3. *Derxia*. Two species are described, *D. gummosa* and *D. indica* (348, 588).

4. *Azotococcus*. Species include *A. agilis* (originally *Azotobacter agilis*).

5. *Azomonas*. Species include *A. macrocytogenes* and *A. insignis* (originally *Azotobacter* species).

Family Enterobacteriaceae: 10 genera, one containing known diazotrophs; anaerobiosis required for $N_2$ fixation.

1. *Klebsiella*. 3 species; $N_2$ fixation may be restricted to *K. pneumoniae*, a species which contains both $N_2$-fixing and non-fixing strains. In a comprehensive analysis of *Klebsiella*, MAHL et al. (448) found that of 31 strains of *K. pneumoniae* tested, 13 fixed $^{15}N_2$. As a result of recent reclassifications (448, 532), $N_2$-fixing species previously designated as *K. rubiacearum*, *Aerobacter aerogenes* (family Enterobacteriaceae), and *Achromobacter* $N_4$ (family Achromobacteriaceae) are now included in *K. pneumoniae*. These reclassifications were made on the basis of serological testing, and the reclassified species subsequently have been shown to possess DNA base ratios typical of *K. pneumoniae* (56.7 to 62.5% GC). Cell-free studies including fractionation of nitrogenase, have been conducted with *K. pneumoniae* M5A.

Family Bacillaceae: Diazotrophs are found in all 3 genera.

1. *Bacillus*. 25 species; anaerobic $^{15}N_2$ fixation was shown for 12 of 13 strains of *B. macerans* and for 15 of 17 strains of *B. polymyxa* (256). Cell-free studies have been made with *B. polymyxa* Hino (232, 257, 726), but similar methods yielded inactive preparations with *B. macerans* (726); however, *B. polymyxa* Hino has a % GC typical of *B. macerans*, not of *polymyxa*, and should probably be considered a *B. macerans* (532).

2. *Clostridium*. 93 species; of 15 tested 13 fixed $^{15}N_2$ (586), including *C. pasteurianum*, perhaps the most studied diazotroph, both *in vivo* and *in vitro*, and the only one for which the metabolic processes supporting nitrogenase are defined (120, 170, 278, 485); of 9 strains of *C. pasteurianum* tested, all fixed $^{15}N_2$, and of 7 strains tested following cell rupture all gave active extracts (726).

3. *Methanobacillus.* $^{15}N_2$ fixation was shown for the only species listed, *M. omelianskii* (559). Initially it was classified as *Methanobacterium*, but tentatively placed in *Bacillaceae* in Bergey's Manual (54). Subsequent work (86) showed it to be a symbiotic association of two bacteria, designated M and S, so classification is still tentative. Both are required for $CH_4$ formation, but it is not established whether both are required for $N_2$ fixation. Recent characterization of the organism designated S indicates it is a new species of bacterium (573).

Order Actinomycetales. Mold-like-bacteria; of 4 families aerobic $N_2$ fixation shown or indicated in two.

Family Mycobacteriaceae. 2 genera with $^{15}N_2$ fixation shown in one.

1. *Mycobacterium.* Nitrogenase activity has been studied extensively *in vivo* and *in vitro* with one species, *M. flavum* (58, 59); $^{15}N_2$ fixation was reported for *M. azot-absorptum* (438); $N_2$ fixation was reported for *M. roseo-album*, *M. sp.* 571 (228, 437) and *M. butanitrificans* (135). Except for *M. butanitrificans*, $N_2$ fixation by these organisms was first established by Russian workers, and they were classified as *Mycobacteria* according to Russian classification methods which were not entirely consistent with the Bergey system. BIGGINS and POSTGATE (60) have examined the question of classification of *M. flavum* 301 and indicate it would most probably be considered a *Nocardia* in the Western system of classification. Classification outside the genus *Mycobacterium* was also supported by an analysis of the respiratory system components of the organism (212).

Family Actinomycetaceae: 2 genera, both tentatively implicated in $N_2$ fixation.

1. *Nocardia.* 45 genera; the indication that *Mycobacterium flavum* 301 may more appropriately be designated a *Nocardia* (see above) lends credence to the early report of METCALFE and BROWN (465) of fixation in this genus, though the type strains failed to fix $N_2$ or $N_2$ $[C_2H_2]$ when retested recently (709).

2. *Actinomyces.* 3 species; endophytes involved in non-legume angiosperm root nodule $N_2$-fixing symbioses are believed to be *Actinomyces*, primarily on the basis of light and electron microscopic observations of nodules (32). No endophytes are positively known, nor has any *Actinomyces* been shown to fix $N_2$, so assignments are still tentative.

## *2. Blue-Green Algae*

Of the approximately 165 genera of blue-green algae in 8 families and 3 orders, $N_2$ fixation has been found in 23 genera, 6 families and 2 orders (Table 2-2). As with the photosynthetic bacteria, vigorous $N_2$ fixation by blue-green algae requires light, though low activity may be observed with some species growing heterotrophically in the dark (223, 687). The long-held belief that $N_2$ fixation is restricted to filamentous species bearing specialized cells called heterocysts, is no longer tenable, though it is clear that these species are dominant among the accredited blue-green algal diazotrophs. Heterocysts are enlarged, thick-walled cells which appear at intervals of 10 to 15 cells in certain filamentous species, and their presence is correlated with the nitrogen nutrition of the organism (516, 684). FAY and LANG have recently compared various methods for the isolation of

Table 2-2. $N_2$ fixation among the blue-green algae

| Order[a] Suborder | Family | Genus and No. of species ( ) implicated in $N_2$ fixation | Evidence for $N_2$ fixation[b] $^{15}N_2$ | $C_2H_2$ | Other[1] | References |
|---|---|---|---|---|---|---|
| Chroococcales (ca. 35 genera) | Chroococcaceae | *Gloeocapsa* (1) | | x | | 247, 732 |
| Chamaesiphonales (ca. 30 genera) | Chamaesiphonaceae | None | | | | |
| | Clastidiaceae | None | | | | |
| Oscillatoriales (ca. 100 genera) | | | | | | |
| Nostocales | Nostocaceae | *Anabaena* (12) | x 3 | x 3 | | 61, 221, 300, 349, 537, 597, 635, 636, 653, 654 |
| | | *Anabaenopsis* (1) | | x | x | 349, 654 |
| | | *Aphanizomenon* (1) | | x 2 | | 661 |
| | | *Aulosira* (1) | | | x | 626 |
| | | *Chlorogloea* (1) | | | x | 222, 224 |
| | | *Cylindrospermum* (4) | | x | x | 349, 654 |
| | | *Gloeotrichia* (1) | x 3 | | | 597 |
| | | *Nodularia* (1) | x | | | 653 |
| | | *Nostoc* (8) | x 3 | x | | 537, 597, 624, 635, 654, 661, 711 |
| | | *Trichodesmium* (1) | x 2 | x 2 | x | 95, 199, 570 |
| | Rivulariaceae | *Calothrix* (5) | ? | | x | 597, 654, 680, 711 |
| | Scytonemataceae | *Scytonema* (2) | | | x | 409, 654, 685 |
| | | *Tolypothrix* (1) | | x | x | 654, 661 |
| | Oscillatoriaceae | *Oscillatoria* (1) | | | x | 459 |
| | | *Plectonema* (1) | x | x 3 | | 221, 664 |
| | | *Schizothrix* (1) | | | x | 702 |
| Stigonematales | Stigonemataceae | *Fischerella* (2) | | | x | 535 |
| | | *Hapolosiphon* (1) | | | x | 408 |
| | | *Mastigocladus* (1) | x 3 | x | | 154, 349, 597 |
| | | *Stigonema* (1) | | | x | 693 |
| | | *Westelliopsis* (1) | | | x | 654 |

[a] Taxonomy according to SMITH, Ref. 633. [b] 1. Growth studies, Kjeldahl N determination or equivalent; 2. cultures not bacteria-free; 3. nitrogenase activity in cell-free preparations

heterocysts from *Anabaena* and have examined the ultrastructure of these cells in detail (225, 412). Heterocysts are more obvious in filaments cultured in the absence of combined nitrogen, but exist as "proheterocysts" in cells growing on ammonium salt, as shown for *Anabaena* (710). In contrast to heterocysts, the "proheterocysts" lack the ability to reduce silver and tetrazolium salts (663). The reductive nature of heterocysts is consistent with the apparent absence of the $O_2$-evolving photosystem II in these cells (683). Their significance remains unclear, though it has been suggested that a reduced species specifically required by nitrogenase is produced in the heterocysts (728); recent evidence suggests that $N_2$ fixation takes place only in these cells in aerobic culture, but occurs in both heterocysts and vegetative cells in anaerobic culture (252). Among the unicellular, non-heterocystous species, $N_2[C_2H_2]$ fixation has been demonstrated only with two strains of *Gloeocapsa*, while other, similar species which were tested did not fix (242, 581, 646, 732). Species of filamentous, non-heterocystous blue-green algae shown to fix $N_2$ or $N_2$ $[C_2H_2]$ include *Trichodesmium* (95, 570) and *Plectonema* (300); in addition, *Anabaena flos-aquae*, which is normally a filamented, heterocystous species, was shown to possess vigorous nitrogenase activity even when cultured in a way in which the heterocysts were < 0.5% of the cell population (405).

Blue-green algae capable of fixing $N_2$ are probably even more widespread than bacterial diazotrophs. Their hardiness and ability to photosynthesize and to form beneficial associations with other microorganisms and higher plant forms (Fig. 2-2) make them extremely adaptable to a wide range of environmental conditions. Accordingly, they are found in both fresh and salt waters, and in terrestrial habitats as well, often growing under extremes of climate (658; p. 58).

## B. Associative Symbioses

A great variety of associations exist between free-living diazotrophs and other organisms (Fig. 2-2), and these appear to profoundly influence $N_2$ fixation. Many such associations have been documented, but probably more exist unnoticed or overlooked. The very nature of the loose associations makes them difficult to detect, since the manipulations of the assay itself are likely to disrupt the symbiosis, particularly if an isolation is involved. Associations involving specific physiological structures, such as root and leaf nodules or cavities, are more readily identifiable.

Most of these symbioses are based on the non-diazotroph contributing carbon compounds to the diazotroph, usually via an exudate, and the diazotroph contributing nitrogen to its host, usually in the form of N-containing compounds generated in the decomposition of dead diazotroph cells. However, the exact nature of many associative symbioses is not always well established, and some may be better characterized as parasitic. Most of the symbioses encourage an increase in the diazotroph population, an effect clearly leading to enhanced $N_2$ fixation. Additionally, the symbiotic relationship may enhance $N_2$ fixation by providing an environment that favors maximal nitrogenase reactivity. In this context there is evidence that the $N_2$ fixation rate of blue-green algae in lichens is enhanced by the low $pO_2$ (p. 102) that is established by the fungal partner (667).

*1. Loose Associations*

Many free-living microorganisms participate in what may be considered loose associations with other organisms. These do not involve structural or morphological accommodation of the $N_2$-fixing organisms, but consist more of close physical contacts in which mutual influence can be exerted between the symbionts.

a) Interactions Among Microorganisms

Probably the simplest associations are those between free-living diazotrophs and other microorganisms. This type of symbiosis is difficult to define with precision because the intensity and nature of the interaction is itself often subtle and transient; however, numerous reports testify to an enhancement in the activity of free-living diazotrophs by microorganisms which do not fix $N_2$. Organisms capable of decomposing polysaccharides have been observed to stimulate $N_2$ fixation by associated *Azotobacter*, *Beijerinckia* and *Clostridium* (226, 345, 346), possibly by supplying utilizable carbon sources. The stimulation of $N_2$ fixation by bacterial diazotrophs associated with certain blue-green algae (536, 616) may occur for the same reason. More subtle effects may be involved in other associations. Dinitrogen fixation by *Mycobacterium* appears to be very sensitive to the presence of other bacteria and is reported to be stimulated by such organisms as *Bacterium*, *Pseudomonas*, and *Flavobacterium* (227, 359). Stimulation may be due in part to the ability of such organisms to reduce the $pO_2$ to a more favorable level (358). KOBAYASHI (393) observed that *Rhodopseudomonas capsulata* required anaerobic conditions to fix $N_2$ in pure culture, but fixed $N_2$ aerobically in mixed culture with *Bacillus megatarium*. *Azotobacter* $N_2$ fixation was significantly increased in the presence of a gram negative rod (543) and a *Pseudomonas* (528), and enhancement of $N_2$ fixation by *Azotobacter* and *Beijerinckia* has been attributed to association with yeasts (184, 189, 590); *Rhizobia* have been reported to enhance $N_2$ fixation by the blue-green alga, *Nostoc* (62). A particularly interesting interbacterial association is suggested in the case of *Methanobacillus omelianskii*, which was initially thought to be a single organism, but was subsequently shown to be a symbiotic association of two bacteria (573).

b) Phylloplane

The phylloplane associations involving free-living diazotrophs and the aerial portion of higher plants have been largely overlooked in $N_2$ fixation studies, yet as early as 1956 it was shown that the leaves of tropical plants generally supported extensive surface populations of microorganisms, among which aerobic diazotrophs were prominent (589). Studies of tropical grasses show that these exude biologically significant concentrations of carbohydrates, but little or no nitrogenous nutrients, thus providing a particularly suitable habitat for diazotrophs (591, 592). Loose phylloplane associations do not appear to be restricted to tropical areas; they have also been indicated in temperate crops (612) and were shown to have the potential for significant contribution to the nitrogen economy of temperate zone woodlands (353).

c) Rhizosphere

Rhizosphere associations, like those of phylloplane, have not yet been extensively studied, though high levels of $N_2$-fixing bacteria

are known to populate the rhizospheres of various plants (587), and evidence for significant nitrogenase activity has been reported for rhizospheres of sugar cane (183), corn (188), rice (188, 745), mangels (362) grasses (184, 481) and marine angiosperms (547). Mycorrhizal associations have been implicated in rhizosphere $N_2$ fixation, but the role of fungi remains ill-defined (68, 69). The high incidence of $N_2$ fixation found with root systems heavily populated with fungal mycelia (578) suggests, in the absence of evidence for mycorrhizal $N_2$ fixation, that these associations are particularly effective in promoting $N_2$ fixation by bacteria and/or algae.

*2. Associations Involving Morphological Modification or Accommodation*

There is one outstanding example of bacterial participation in this more intimate type of association. This is the leaf-nodule symbiosis in which high concentrations of the $N_2$-fixing facultative anaerobe, *Klebsiella*, occur extracellularly in the crude subepidermal leaf protuberances of the tropical plant, *Psychotria* (421). These bacteria appear to be involved in the hormonal, as well as nitrogen, metabolism of the plant (620). Nodulated leaves were recently found unable to reduce $C_2H_2$ to $C_2H_4$ (619), possibly because photosynthetically generated $O_2$ inhibited nitrogenase activity; this point could be tested by conducting analyses in the dark. Leaf nodule symbioses have been indicated for other plants, particularly *Pavetta* and *Ardisia*, but the evidence for $N_2$ fixation in these associations remains equivocal (313).

The blue-green algae are known to form associations of varying complexity with representatives of virtually all other plant forms. Diazotrophs have been reported to occur intracellularly in other algae, including a green alga (410) and diatoms (239). Among the bryophytes, *Nostoc* species are found in symbiosis with the liverworts *Blasia* and *Cavicularia*, where they occupy cavities in the underside of the thallus, and a *Hapalosiphon* has been implicated in an association with sphagnum moss (654). Incorporation of $^{15}N_2$ has been shown with the *Nostoc* associated with *Blasia* (70). An *Anabaena* species and the water fern *Azolla* form a symbiosis in which the alga is housed in leaf pores (411).

The symbiosis between *Gunnera* and *Nostoc*, in which the *Nostoc* are localized in wart-like structures at the leaf bases, is the only reported example of a blue-green alga-angiosperm association which involves specialized structures (624). A form of root nodule symbiosis occurs with high incidence between certain tropical gymnosperms of the family Cycadaceae and blue-green algae identified as *Anabaena* or *Nostoc* (9, 68, 658). These symbioses are qualitatively unlike the angiosperm root nodule symbioses (p. 28), since the nodules appear to form even in the absence of endophyte; more importantly the symbioses are associative rather than obligatory. The details of infection are unknown and the relationship between endophyte and host appears less intimate than in the angiosperm root nodules since the algae do not appear to actually penetrate host cells, but rather become embedded in the areas between cells. Nodulation is reported for eight of the nine genera in Cycadaceae, including *Cycas*, *Certozamia*, *Encephalartos*, *Macrozamia*, *Stangeria*, *Bowenia*, *Zamia* and *Dioon*; about one third of the approximately 90 cycad species are involved (32). Root nodule symbiosis has also been reported to occur in a second family of gymnosperms, Taxaceae, specifically in the genus *Podocarpus*, but the reports of $N_2$ fixation are conflicting (28, 29, 43). The endo-

phyte, which has been suggested to be fungus (29), does not appear to be necessary for development of nodules (380).

Perhaps the most intimate involvement of free-living diazotrophs with other organisms is found in the lichens (379, 470, 658). Species of *Nostoc* and *Calothrix* have been identified. In numerous lichens, including *Lichina* species (658), *Peltigera rufescens* and *Collema tunaeforme* (304), *Leptogium lichenoides* and *Collema granosum* (70), $^{15}N_2$ incorporation by the phycobiont, *Nostoc*, has been observed; in *Peltigera aphthosa*, $^{15}N_2$ fixed by the Nostoc phycobiont was shown to be utilized by the mycobiont *(Coccomyxa)* (379).

### *3. Associations with Animals*

The scope of the participants in $N_2$-fixing associations has recently been extended beyond the limits of the plant kingdom. Involvement of higher animals is becoming increasingly well documented in reports of $N_2[C_2H_2]$-fixing activity by the intestinal flora of both ruminants and non-ruminants. The animals which have been implicated include swine, and guinea pig (49), human (14, 49), reindeer and goat (255), sheep (201, 255, 563, 564), steer (201, 285) and camel (201). It appears that a *Klebsiella* may be involved in the $N_2$ fixation found in human and swine fecal matter (49).

## II. Obligatory Symbioses

### A. Non-Legume Angiosperms

These root nodule symbioses are sometimes referred to as alder-type, indicative of the most studied member of the group. They share many characteristics with legume symbioses, but possess unique qualities which clearly warrant separate classification. They are even less similar to the gymnosperm and leaf nodule symbioses (p. 27). Recent dissertations which deal with the alder-type symbioses include chapters by BOND (68) and BECKING (32) and a book by STEWART (654).

Symbioses are known among 14 genera of angiosperms in 8 families and 7 orders (Table 2-3); about one third of the over 300 species are reported to be nodulated and probably $N_2$-fixing, but the actual fraction may be greater because many species have not been examined. Though the taxonomy of the angiosperm genera indicates a phylogenetic relationship, BOND (68) suggests that the relationship may be simply temporal, the result of a high incidence of this type of symbiosis at a propitious time in evolution. Non-legume angiosperm root nodule $N_2$-fixing symbionts have world-wide distribution and are particularly conspicuous in marginal areas where vegetation is not well established. Certain genera, such as *Alnus, Coriaria, Elaeagnus,* and *Hippophaë,* are found the world over, while others such as *Ceanothus* and *Shepherdia* have more limited distribution (68).

Probably the most elusive aspect of the alder-tpye symbioses is the identification of the endophyte. Numerous claims have appeared in the literature (see Ref. 32), but none has as yet been independently confirmed. At present it appears that no organism has been reproducibly isolated, cultured and shown to form effective nodules. The most commonly observed microorganisms in the nodules are considered to be actinomycetes; three morphological forms are frequently

noted: hyphae, vesicles and bacteria-like cells (32, 244). BECKING (30) has grown such isolates in callus tissue of alder nodule origin, but callus macerates produced only occasional and ineffective nodules when applied to sterile alder seedlings. Inoculation of sterile seedlings is normally accomplished with relative ease using crushed nodules.

Table 2-3. Non-legume angiosperm symbioses (after BOND, Ref. 68)

| Order | Family | Genus | Species | |
|---|---|---|---|---|
| | | | Total | Known Nodulated |
| Coriales | Coriariaceae | *Coriaria* | 15 | 12 |
| Myricales | Myricaceae | *Myrica* | 35 | 11 |
| | | *Comptonia* | | |
| Fagales | Betulaceae | *Alnus* | 35 | 25 |
| Casuarinales | Casuarinaceae | *Casuarina* | 45 | 14 |
| Rhamnales | Elaeagnaceae | *Elaeagnus* | 45 | 9 |
| | | *Hippophaë* | 1 | 1 |
| | | *Shepherdia* | 3 | 2 |
| | Rhamnaceae | *Ceanothus* | 55 | 30 |
| | | *Discaria* | 10 | 1 |
| Ericales | Ericaceae | *Arctostaphylos* | 40 | 1 |
| Rosales | Rosaceae | *Dryas* | 4 | 1 |
| | | *Purshia* | 2 | 2 |
| | | *Cercocarpus* | 20 | 1 |
| | | | > 300 | 110 |

Until the endophytes are identified by successful re-inoculation tests and studied in independent culture, it is unlikely that these symbioses can be positively categorized either as obligatory associations or as more casual associations. Numerous similarities between alder-type and legume symbioses suggest the former (32, 68). Re-inoculation studies with crushed nodules have established that cross inoculation groups exist in alder-type symbioses, just as with legumes. The nodule initiation and development processes in the alder-type symbioses follow a pattern very similar to that described for legumes, including root hair curling, entry by infection thread through the root hair, probable preferential invasion of polyploid cells, and the eventual appearance of bacteroid-like bodies (p. 32).

Though numerous similarities exist there are also distinct differences. Most obvious is the difference in nodule anatomy (654). The alder-type nodules have a characteristic structure consisting of a central conducting element surrounded by cortex tissue and finally an outer cork layer (Fig. 2-3). The pattern of infected cortex cells seems to vary from a series of concentric zones in *Alnus* to a rather random distribution through the cortex in *Myrica*. In contrast, the vascular system in legume nodules is external to the infected area, which occupies the entire central portion of the nodule. A second major difference between alder-type and legume nodules is the apparent lack

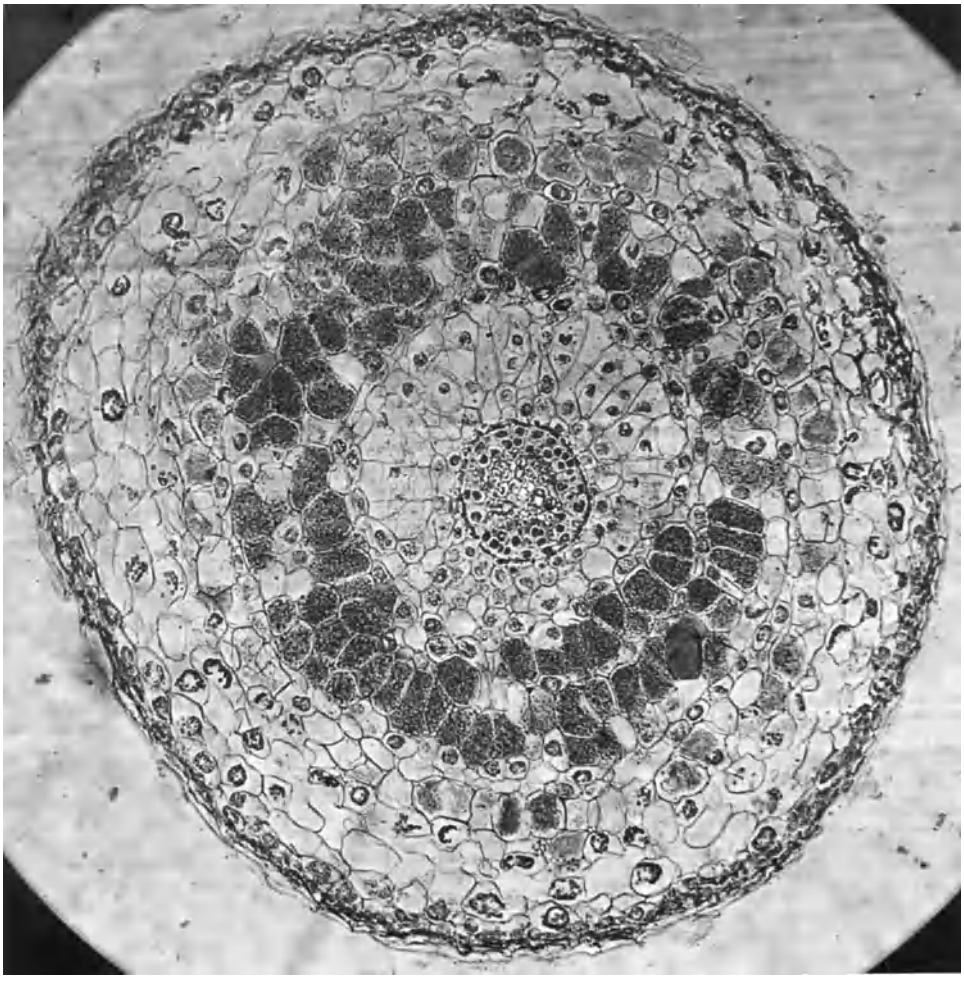


Fig. 2-3. Cross section of *Alnus glutinosa* root nodule (X 600); after BECKING (Ref. 31)

of leghemoglobin in the former. Though DAVENPORT (153) reported its presence in several alder-type nodulated species based on an absorption band at 563 nm, others were unable to confirm this (see Ref. 32). Alder-type nodules, however, do show interior red coloration, but this has been attributed to anthocyanins or similar pigments (32, 67).

## B. Legumes

The family Leguminoseae comprises 12,000 to 14,000 species of 700 genera and 2 subfamilies, making it one of the largest groups of plants (654). Its members vary in form from large woody perennials to low lying ground cover and include many major agricultural crops. The exact incidence of $N_2$ fixation among them is unknown, but nodulation, which is indicative of fixation, occurs on more than 90% of the subfamilies Mimosoideae and Papilionatae, and on about one-third of the subfamily Caesalpinioideae (Table 2-4).

The legumes are most prevalent in the tropics, which is believed to be their place of origin, but the Mimosoideae and Papilionatae have become widespread in temperate areas as well. The 100 to 200 agriculturally important legumes have a distribution pattern similar to that of man. These are listed among the Papalionatae and include such plants as soybeans, groundnuts (peanuts), lentils, peas, beans, and other pulse plants, and forage crops such as alfalfa (lucerne) and clover.

Table 2-4. Incidence of $N_2$-fixing symbiosis among legumes (after ALLEN and ALLEN, Ref. 9)

| Subfamily | Genera | | | Species | |
|---|---|---|---|---|---|
| | Total No. | No. examined | Nodulated (%) | No. examined | Nodulated (%) |
| Mimosoideae | 30 | 19 | 95 | 130 | 92 |
| Caesalpinioideae | 91 | 31 | 48 | 97 | 34 |
| Papilionatae | 308 | 154 | 97 | 969 | 94 |
| Total | 429[a] | 204 | 89 | 1196 | 89 |

[a] More current estimates place this total at about 700 genera

The importance of legumes in agriculture has prompted extensive investigation of the *Rhizobium*-legume symbioses at all levels, extending from agronomy to biochemistry. Recent reviews of legume symbioses include those by DIXON (182), NUTMAN (526) and BERGERSEN (47), and the subject is discussed in detail by STEWART (654). Such aspects as growth and physiology of the *Rhizobia*, *Rhizobium*-legume specificities and the physiology of the infection process and nodule development have become increasingly well understood, though an explanation of legume symbiosis at the molecular level remains to be defined. The recently achieved *Rhizobium*-soybean tissue culture symbiosis (314), discussed below, may prove a useful tool in the pursuit of this problem.

### *1. Rhizobia*

The qualifying characteristic for this genus of soil bacteria is the ability to form an $N_2$-fixing symbiosis with a legume. It was observed early that positive interactions between bacterium and plant followed definite patterns of specificity such that certain varieties and strains of *Rhizobia* formed effective symbioses only with certain legumes. These specificities formed the basis for the categorization of the legumes and *Rhizobia* into 16 cross-inoculation groups by FRED, BALDWIN and McCOY (238). It was believed that these groups were mutually exclusive, but promiscuity (ability to infect plants of more than one cross-inoculation group) among the *Rhizobia* has since been amply demonstrated (347, 413, 522), and the utility of such a classification system is now questionable. Taxonomic division along metabolic lines has been suggested which would recognize the rapid-growing acid-producing *Rhizobia* as one group and the slow-growing alkali producers as a second group (192, 413). Other classifications suggest a very complex taxonomy for these bacteria. Numerical categorization according to coincidence of characteristics (254, 478)

indicates that the fast-growing strains are more closely related to the *Agrobacteria* than to the slow-growing *Rhizobia*; DNA base-ratio analyses and hybridization studies have led to the same conclusion (157, 158, 161, 302), but a recent analysis of characteristics indicates some distinct metabolic differences between the *Rhizobia* and *Agrobacteria* (629). The taxonomic position of bacteria which can induce nodulation but fail to establish an $N_2$-fixing system is unclear. Minor metabolic variations may be significant in the determination of effectiveness, as indicated in a recent report (608) that a mutant of *R. trifolii* required riboflavin for effectiveness; in the same vein, it was observed (611) that effective *Rhizobia* strains synthesized higher levels of riboflavin and cobalamine than ineffective strains, and strain selection on that basis was suggested.

The *Rhizobia* are widespread in soil, where they grow saprophytically on combined nitrogen. Attempts to induce nitrogenase in free-living *Rhizobia* have been uniformyl unsuccessful (47). They are normally aerobic, but have been cultivated anaerobically with nitrate as electron acceptor (see Ref. 47). The strains which infect agriculturally important legumes are cultured commercially, and it is common agronomic practice to inoculate seed with these organisms at time of sowing.

Infection and nodule development take place in the temperature range $7^{o}$ to $33^{o}$, optimally at about $30^{o}$ (246); NUTMAN et al. (527) observed that holding day-old clover seedlings at $3^{o}$ caused an increased rate of infection when the temperature was subsequently raised to $19^{o}$. Calcium is required for nodulation with the need apparently most critical during the initial stages of infection (436). Molybdenum, a component of nitrogenase, is also required for effective nodules, though this may be replaced by tungsten, but not vanadium, according to recent work with lucerne (350). A cobalt requirement for effective legume symbiosis has been known for some time (163, 215), and more recently a specific $B_{12}$ coenzyme dependency has been domstrated for the ribonucleotide reductase of *Rhizobium* species and bacteroids (136). Beneficial effects of sulfur (55) and zinc (593) have been noted, and inhibition by fixed forms of nitrogen, particularly nitrate, is well known (152, 182). Variable effects have been observed following plant hormone applications (557, 676). Total nodule mass is determined by the plant when the *Rhizobia* are abundant and presumably is regulated at least in part by an inhibitor which first-formed nodules produce to block additional nodulation (182, 654). Nodulation may also be subject to control by phytochrome, the blue-green regulatory protein which is implicated in many plant growth responses. LIE (424) has shown a typical phytochrome effect in which far-red light inhibited nodulation in peas and beans, and red light reversed this inhibition. The effects appear to be direct, but the systems are too complex to conclude this with complete assurance. Soil components including gallic and tannic acids and certain leaf exudates have been found to limit nodulation in some cases (64, 575).

### *2. Establishment of an Effective Legume Symbiosis*

This is accomplished according to the following chronology; 1. infection of the host root cell by *Rhizobia*, 2. development of an infection thread into the cortex tissue of the host, 3. proliferation of cortex tissue to form a nodule, 4. deposition of the *Rhizobia* into the host cell and formation of vesicle membranes around them, 5. proliferation of *Rhizobia* to the bacteroid state. The sequence is illustrated in

Figure 2-4, and the morphology of a developing nodule is shown in Figure 2-5.

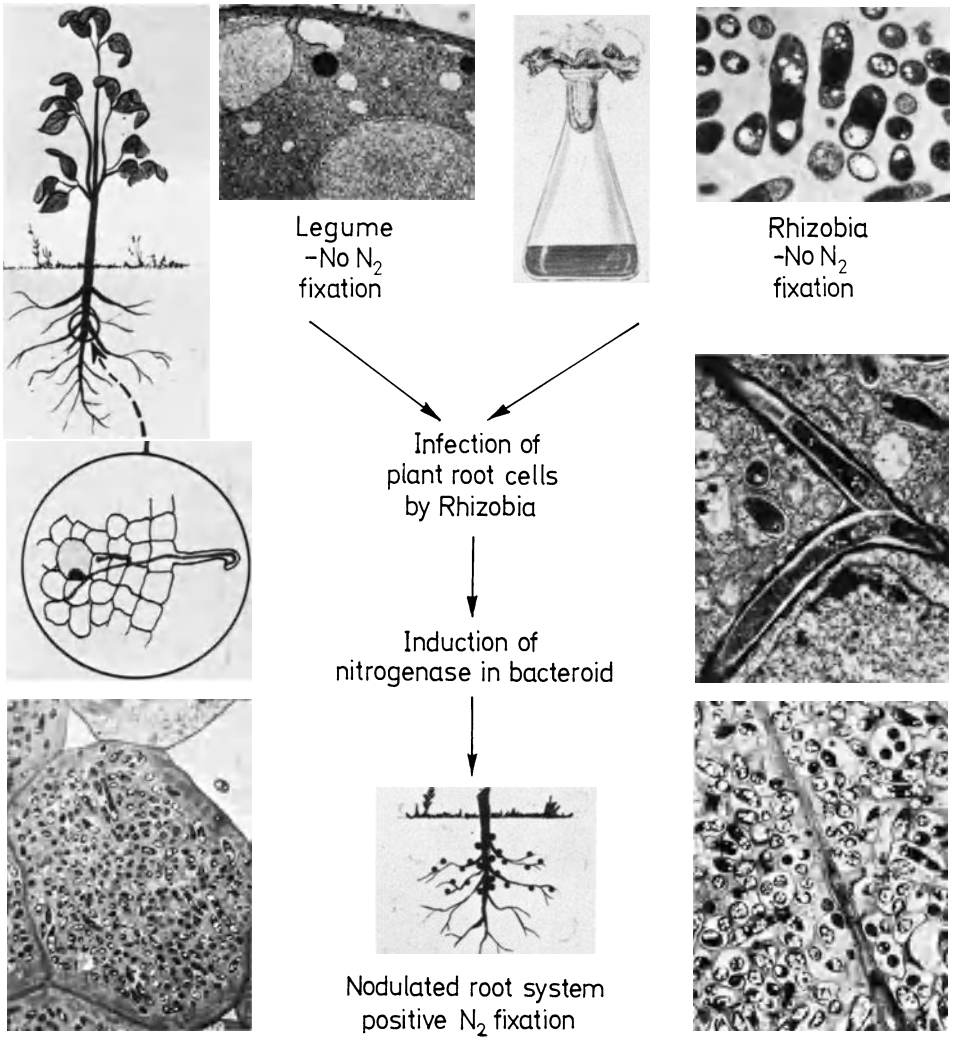



Fig. 2-4. Establishment of legume symbiosis. Infection thread penetration through root hair cell and into cortex cell is sketched at center, left, and shown in electron micrograph at center right; dense population of bacteroids in cortical cells, typical of the mature symbiosis, is shown in micrographs at bottom

Free-living *Rhizobia* in the soil are observed to move preferentially toward the root hairs of developing legumes, apparently in response to an undefined attractant excreted by the root. In the normal infection sequence, the root hair curls in response to the dense rhizobial population which aggregates at its surface; the curling reaction is possibly induced by a rhizobial extracellular polysaccharide (320) or by indole-acetic acid produced by the bacteria or by the

host under bacterial influence; possible involvement of gibberellin has also been suggested (182). Though curling is common, it is not a mandatory event and in fact cannot occur either in aquatic legumes or in the *Rhizobium*-tissue culture symbiosis, both of which lack root hairs. The root hair cell wall becomes weakened, possibly in response to the activity of polygalacturonase which is thought to be produced extracellularly by the host in response to bacterial stimulation, though there is controversy concerning this mechanism (425, 434, 445). The *Rhizobia* then gain entry through the weakened cell wall of the curled root hair. If only a single *Rhizobium* enters, the resulting nodule will contain a genetically homogeneous population of bacteroids; in contrast, infection by more than one bacterium can lead to genetic heterogeneity. Entry appears to occur via invagination (524, 595) of the host cell wall, there being no evidence for physical penetration through this barrier.

Upon entry of the *Rhizobia* into the root hair, they become enclosed in a structure called the infection thread which grows from the site of invagination. This infection thread is essentially a cellulose tube which is continuous with the host cell wall and grows through the root hair cell and cortical cells of the developing nodule. A close physical relationship between the infection thread and the host cell nucleus is apparent. Invagination invariably occurs at a point proximal to the host cell nucleus, which is enlarged relative to the nuclei of uninfected cells; the nucleus then accompanies the infection thread down the root hair into the main body of the root hair cell. As the infection thread proceeds through other cells, it normally passes close to their nuclei. Coincident with the development of the infection thread and the passage of *Rhizobia* through it, stimulation of cortical cell division occurs and the proliferation of these cells results in formation of the nodule. Cytokinins are implicated in this response, and the production by *Rhizobia* of a material with cytokinin activity has been demonstrated (558, 688). Abscissic acid on the other hand inhibits cortical cell division and thus suppresses nodulation (557).

Termination of the infection thread and deposition of the *Rhizobia* appears to occur preferentially in polyploid host cells, which frequently occur in the nodular tissue, but the precise nature of the deposition mechanism is not entirely clear and may vary among legumes. The process in numerous species has been examined by electron microscopy. In soybean the release mechanism was observed to involve a lateral widening of the infection thread in the host cell, aggregation of *Rhizobia* in this bulge, extension of the bulge by synthesis of cellulose-free membrane, attachment of bacteria to this membrane

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Fig. 2-5. Longitudinal section of a nodule on 15-day-old clover seedling in sand culture inoculated with *R. trifolii* strain 205. Fixed in Flemming's solution (medium); section 5μ thick; stained with Heidenhain's hematoxylin. Camera lucida drawing; approx. x 175 (Ref. 238).

*A* Cells of the root cortex, stretched and broken by the swelling of the nodule. *B* Uninfected meristematic cells in the growing end of the nodule. *C* Fragments of infection threads. *D* Bacteria free in the host cells; coccoid and rod forms in recently infected cells and progressively more vacuolate and swollen forms in the older infected cells or bacteroid region. *E* Hypertrophied and deformed nucleus showing conspicuous nucleolus and chromatic granules. *F* Uninfected cells. *G* Hypertrophied plant cell with large vacuole. *H* Vascular strands laying in the cortex of the nodule and extending to the vascular system of the root stele. *I* Strands of vascular system of the root showing junction with vascular strands of the nodule

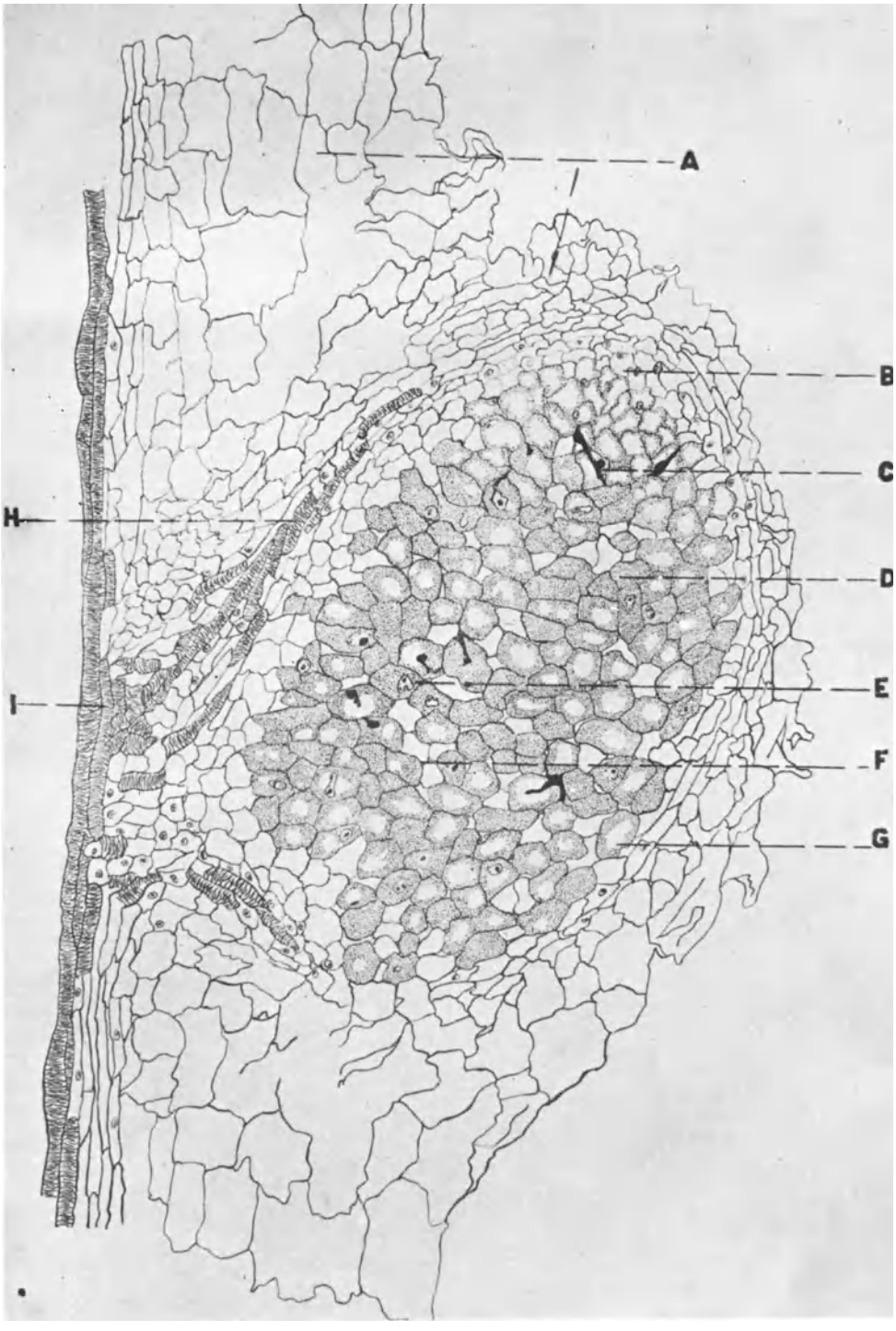


Fig. 2-5

and finally enclosure of the individual bacteria in membrane structures which disengaged from the thread and became independent bodies in the host cell cytoplasm; the release process was likened to endocytosis (250). In clover the *Rhizobia* were reported to emerge from the thread into host cell cytoplasm where they were immediately enclosed by cytoplasmic membrane of host cell origin (182, 496). In *Phaseolus* the *Rhizobia* appeared to remain as free-floating bacteria in the host cytoplasm and vacuole for an extended period of time following release through the dissolved infection thread terminus, eventually becoming enclosed in membranes which originated from the outer layer of the host cell nucleus. Prior to the release of *Rhizobia* from the infection thread these membranes were observed to form initially as blebs on the nuclear membrane which then disengaged and subsequently enclosed the free-floating bacteria (566). In lupin the endoplasmic reticulum of the host cell has been suggested as the origin of the membranes which form the bacterial enclosure (354).

Proliferation of the bacteria within the membrane enclosures, or vesicles, occurs in some cases by division of both bacteria and membrane to give increasing numbers of enclosures containing a single organism; in other cases, and particularly in the latter stages of proliferation, the membrane does not divide, but the enclosed bacteria undergo several replications to give vesicles containing up to about eight organisms. Lateral fusion of vesicles has been reported to occur in peas and clover nodules as these become increasingly crowded in the nodular tissue (263). Proliferation continues until the host cell is almost filled with vesicles and the remaining cytoplasm is restricted to the periphery of the cell. BERGERSEN (43) has observed that an average soybean root nodule contains about 35,000 central tissue cells each of which contains up to 100,000 vesicles within each of which are 4-6 bacteroids.

During this period of maturation the bacteria undergo important changes which culminate in their conversion to physiologically and biochemically altered forms known as bacteroids. The most notable biochemical change is the development of an ability to reduce $N_2$, presumably an immediate result of *de novo* synthesis of nitrogenase. In addition changes in respiratory enzymes (358) and in cytochrome and other heme-protein composition occur within the microorganisms (17, 18), and large amounts of leghemoglobin are synthesized (p. 164). The localization of leghemoglobin remains controversial, but is definitely exterior to the bacteroids. BERGERSEN (42) and DILWORTH and KIDBY (175) concluded that leghemoglobin is within the vesicular membrane, while DART (150) reported evidence favoring its location exterior to the vesicle. The amount of leghemoglobin is positively correlated with the amount of nodular $N_2$ fixation (130); in the developing nodule the leghemoglobin content increases as $N_2$ fixation increases, but the rate of leghemoglobin increase is more than 10 times greater (283). Leghemoglobin has been shown to facilitate the diffusion of $O_2$ in aqueous media (331, 725), and it presumably functions in this capacity physiologically, though its function has not yet been established unequivocally. Possible roles include supplying $O_2$ for nodule respiration or, alternatively, protecting nitrogenase by scavenging $O_2$. Direct function in nitrogenase catalysis is precluded by the lack of a leghemoglobin requirement for nitrogenase activity by bacteroid preparations (315).

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Fig. 2-6. Electron micrographs of soybean root nodule from onset of infection by *Rhizobium*, through peak of $N_2$-fixing activity, and into sensescence. First micrograph was taken 12 days (d) after planting

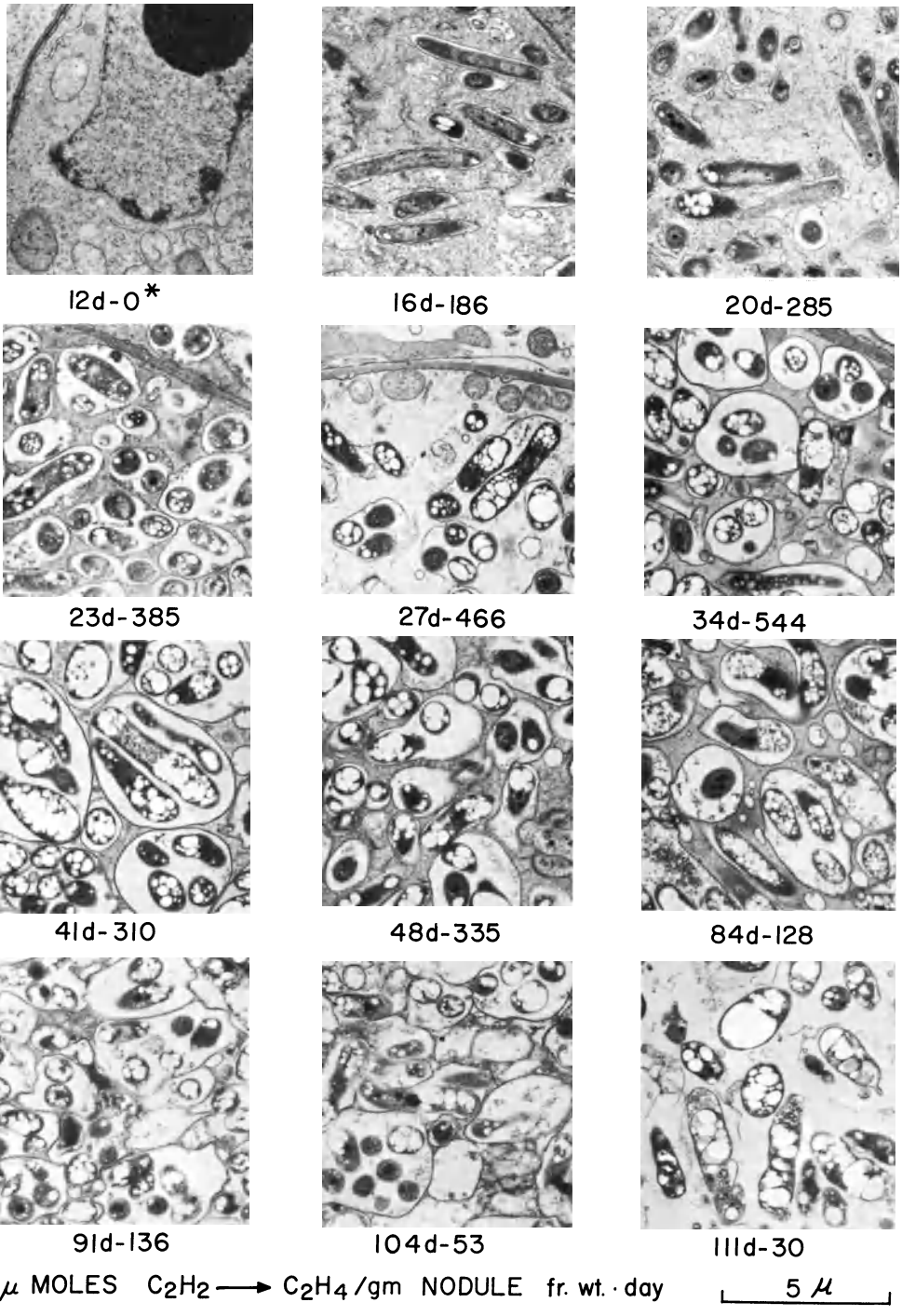
12d-0*
16d-186
20d-285
23d-385
27d-466
34d-544
41d-310
48d-335
84d-128
91d-136
104d-53
111d-30
*μ MOLES $C_2H_2$ ⟶ $C_2H_4$ /gm NODULE fr. wt. · day
5 μ

Fig. 2-6

Physiologically, the most important change in *Rhizobia* appears to be conversion from a vegetative to a non-vegetative state. This distinction has been used as a basis for distinguishing bacterium from bacteroid, but the precise point at which a bacterium becomes a bacteroid is not well established, nor is the bacteroidal state itself very well defined in the literature. Dinitrogen fixation, formerly considered to be an exclusive property of the bacteroid or non-dividing form, now appears to occur also in vegetative or dividing *Rhizobia* within the nodule (47, 283). The correlation of $N_2$-fixing activity and soybean nodule morphology has been examined (Fig. 2-6), and it was found that activity increased from zero at twelve days after planting to 500 μmoles $C_2H_2$ reduced/g nodule fresh weight × day at 35 days; corresponding increases occurred in bacteroid number per vesicle and inclusions per vesicle; at senescence the loss of activity paralleled dissolution of the vesicles (283, 315).

*3. Tissue Culture Symbiosis*

In an application of tissue culture techniques to legume symbiosis, HOLSTEN et al. (314) recently described conditions for effective *Rhizobia* infection of callus tissue of soybean root origin. The resulting symbiosis provides a possible test system for investigating various facets of the legume symbiosis with minimal interference from plant influences. At present the $N_2[C_2H_2]$-fixing activities obtained with the infected callus tissue are a maximum of about 1% of those recorded for intact nodules, and the application of this system will probably be limited until higher activities are developed. In the work already reported negative effects on development of symbiosis were readily correlated with additions of auxin and cytokinin (i.e., coconut milk) and with light. The morphological changes which accompany the onset of nitrogenase activity in the callus tissue were found to closely parallel the changes observed in intact nodule systems. Electron micrographs revealed the presence of a structure similar in appearance to an infection thread and also showed some of the intracellular bacteria to be enclosed in membranous sheaths or vesicles. With time, infected callus cells displayed the same dense population of microorganisms typical of intact nodules.

## Concluding Remarks

In recent years the categorization and characterization of the diazotrophs have progressed on an increasingly broader scale as more and more investigators have become involved. New developments and understanding have been generated in this intensified effort, and these in turn have stimulated and directed additional work. Of the pertinent technological developments, the advent and widespread use of the acetylene reduction assay have had the greatest impact. Important concepts which have arisen and gained momentum in recent times include new appreciation for the importance of $pO_2$ effects on $N_2$ fixation *in situ* and the increasing awareness of the ubiquity of associative $N_2$-fixing symbioses in nature. Recognition of the latter owes much to yet another recent development, the spread of systematic studies of $N_2$ fixation and diazotrophs to tropical climates. While these associations are not restricted to the tropics, certainly they are prevalent there, and they came into focus as the geographic base of study expanded more intensively into the tropical areas. As a result of the over-all increased effort in investigations of the diazotrophs, it is now possible to list and characterize these organisms with more confidence than ever before. Much of course remains to be done; however, it appears that for the first time the means are at hand to complete the tasks still undone.

# Perspectives

## I. Evolution

A chronology for the evolution of major processes in nitrogen metabolism, culminating in the capacity to fix $N_2$, is developed in this section. It is based on a consideration of the composition of the primordial atmosphere and on a logical sequence of evolutionary challenge and response, proceeding through the following successive stages 1. initial utilization of ammonia, 2. oxidation of ammonia to nitrate, 3. conversion of nitrate to $N_2$ and $N_2O$, as well as reconversion of nitrate to ammonia, and 4. fixation of $N_2$ to ammonia (Table 3-1). Evolution of nitrogenase in free-living organisms is discussed first, followed by proposals for associative and obligatory symbioses.

Table 3-1. A possible evolutionary sequence leading to nitrogenase

| Stage | Challenge | Response | Nitrogen Utilization Capacity of Organisms |
|---|---|---|---|
| 1 | $NH_3$ abundance | $NH_3 \rightleftarrows$ cellular N | $NH_3 \rightleftharpoons$ cellular N |
| 2 | $O_2$ appearance | $NH_3 \xrightarrow{[O]} NO_3^-$ + energy | $NH_3 \rightleftharpoons$ cellular N<br>$\downarrow$<br>$NO_3^-$ |
| 3 | $NO_3^-$ accumulation | $NO_3^- \xrightarrow{[H]} NH_3$<br>$NO_3^- \quad e^- \quad N_2$+ energy | $NH_3 \rightleftharpoons$ cellular N<br>$\uparrow\downarrow$<br>$NO_3^- \longrightarrow N_2$ |
| 4 | $N_2$ accumulation | $N_2 \xrightarrow{[H]} NH_3$ | $NH_3 \rightleftharpoons$ cellular N<br>$\uparrow\downarrow$ $\searrow$<br>$NO_3^- \longrightarrow N_2$ |

### A. $N_2$ Fixation in Free-Living Organisms

It is generally accepted that life on earth originated under a reducing atmosphere, following a long period of abiogenic organic chemical evolution (378, 529 and references therein). The primordial atmosphere presumably contained $CH_4$, $NH_3$, $H_2O$, and to a lesser extent $H_2$, $N_2$, $H_2S$, CO and $CO_2$. Dioxygen was present initially in only trace amounts, generated via the photolysis of water and rapidly consumed in reactions with the wide range of available reductants. Through the millenia this reactivity became the driving force in the synthesis of new molecules, and these gradually changed the character of the environment as more and more oxidized components accumulated.

The earliest successful life forms were unquestionably simple anaerobic organisms whose metabolic processes were directed by the nutrients

most abundant and accessible. With regard to nitrogen, ammonia must be considered as the major available form, and indeed the almost universal capacity of organisms to utilize ammonia attests to a deep evolutionary root favoring this compound. With ammonia available the ability to utilize $N_2$ would confer no selective advantage, and it is unlikely that nitrogenase developed so long as ammonia was plentiful.

The primordial environment would have readily supported a balance between the facilely interchangeable, free ammonia and organically bound reduced nitrogen. This simple cycle presumably came under increasing pressure as the character of the atmosphere gradually became less reductive, and as the $pO_2$ began to increase, an alternate, oxidative, route for ammonia became possible. Thus, organisms developed with a capacity to oxidize ammonia and to utilize the energy of this oxidation. Numerous existing bacteria are known to catalzye the reaction,

$$NH_4^+ + \tfrac{3}{2} O_2 \rightarrow NO_2^- + 2H^+ + H_2O$$

and others to catalyze the reaction,

$$NO_2^- + \tfrac{1}{2} O_2 \rightarrow NO_3^-.$$

Collectively, these reactions are referred to as nitrification (p. 48). In the early environment their effect was to amass nitrate at the expense of ammonia.

Two different metabolic mechanisms appear to have developed in response to the challenge of nitrate accumulation. These mechanisms, both found in existing microorganisms, have been called assimilatory and dissimilatory nitrate utilization (p. 48). The former involves reduction of nitrate to ammonia, which is then incorporated into cellular components; the latter utilizes nitrate or nitrite as an electron acceptor in energy-yielding reactions which produce $N_2$ and to a lesser extent $N_2O$:

$$2NO_3^- + 12H^+ + 10e^- \rightarrow N_2 + 6H_2O$$
$$2NO_3^- + 10H^+ + 8e^- \rightarrow N_2O + 5H_2O.$$

As with ammonia utilization, the widespread ability of organisms to utilize nitrate reflects the evolutionary significance of this nitrogen compound. The proposed existence of a molybdo-protide common to nitrogenase and nitrate reductase, suggested recently (515), would support an evolutionary link between the two enzymes, but which might have come first is a matter of conjecture.

Dissimilatory nitrate utilization in which nitrate is reduced beyond nitrite to $N_2$ or $N_2O$ is also called denitrification. It is likely that this mode of nitrate consumption developed earlier than the assimilatory mode. Presumably the assimilatory mechanism would not develop until the pressures of ammonia depletion became excessive. On the other hand, the dissimilatory reactions represented potential routes to new sources of biological energy, a relatively rare evolutionary opportunity in the still primarily reductive environment. Considering the competitive advantage of a new energy yielding reaction, it is reasonable to assume that an adaptive response was not long in coming. As this reactivity proliferated, metabolically inert $N_2$ began to accumulate in the atmosphere.

Loss of nitrogenase in some species might have resulted simply from an inability to perfect a mechanism to protect nitrogenase from $O_2$.

In the evolutionary sequence described above, it is assumed that nitrogenase developed in response to a crisis in nitrogen nutrition. It is possible, however, that the enzyme could have arisen under circumstances quite unrelated to the function of providing metabolic nitrogen, and therefore at an earlier time than has been indicated. For example, if the known nitrogenase substrates are compared with compounds present in the primordial environment it is apparent that toxic nitriles such as cyanide are common to both groups; accordingly it is not unreasonable to think that nitrogenase may have first developed as a detoxifying mechanism. A second possibility is that the enzyme developed simply as a means to cope with triple bonds. The gradual disappearance of the substrates whose challenge had created the enzyme would have overlapped the gradual increase in $N_2$ accumulation, so that the advent of $N_2$ fixation would represent little more than a substrate change for an existing enzyme. Another possibility, suggested by PARKER (542), is the development of nitrogenase as an enzyme to utilize $N_2$ in an $N_2$-based respiration, as cytochrome oxidase utilizes $O_2$ in $O_2$-based respiration. The co-occurrence of hydrogenase with nitrogenase argues against such a role for nitrogenase, however, since hydrogenase provides a ready outlet for electrons; furthermore no indication of such a function remains in the $N_2$-fixing characteristics of any known existing diazotroph. If IMSHENETSKII is correct in considering *Clostridium* the primitive prototype diazotroph, then nitrogenase may have originated earlier than the chronology developed above indicates; alternatively, the ability to fix $N_2$ could have developed in the genus long after the genus itself emerged.

## B. $N_2$ Fixation in Symbioses

Between the extremes of a simple free-living $N_2$-fixing bacterium and the highly developed legume symbiosis, there is a very wide range of interorganismal relationships involving diazotrophs and other organisms in associations of varying complexity (p. 25). The development of such associations would appear to be a likely consequence of natural selection in view of the survival value of the ability to fix $N_2$. In return for supplying ammonia the $N_2$-fixing partner may receive such benefits as a carbohydrate supply, extension of its ecological range, or a diminished $pO_2$ in which to function more efficiently, as has been indicated for lichens (379) and for *Mycobacterium* (358).

The legume root nodule symbioses are generally regarded as the most recent evolutionary development of $N_2$ fixation and are among the most intimate symbioses found in nature. The intimacy is such that neither nitrogenase nor leghemoglobin can be synthesized by the symbionts independently; in the case of leghemoglobin it has been shown that while apoleghemoglobin synthesis takes place in the nodule cell cytoplasm and is controlled by plant DNA, the heme portion is synthesized within the bacteroid (140, 172, 173).

PARKER (544) and DILWORTH and PARKER (176) suggest that the obligatory symbiotic relationship of the legumes is an extension of an associative type of symbiosis in which the bacterium, originally capable of free-living $N_2$ fixation, was better able to survive during its free-living period by disencumbering itself of the genetic machinery needed for a nitrogenase system. During this free-living period the bacterium would presumably exist in an environment rich in nitrogen from the decaying legume, and an $N_2$-fixing system would provide no advantage.

Again a critical situation can be envisioned, and under the pressure of nitrogen starvation the capacity to reduce $N_2$ to ammonia presumably developed at that time. With the establishment of $N_2$ fixation, life forms collectively became equipped to cope with the major nitrogen compounds in the environment, and the basic requirements for the biocycling of nitrogen were met. The threat of nitrogen starvation no longer remained a vital factor in evolution.

BURRIS (109) has considered possible evolutionary relationships among the diazotrophs and places the probable origin of $N_2$ fixation in a photoautotroph, most likely the antecedent of present day green sulfur bacteria. From this source it is suggested that the other photosynthetic bacteria, the non-photosynthetic sulfur bacteria, and eventually the blue-green algae evolved. The *Clostridia*, because of their requirement for complex organic substrates, were suggested to follow the photosynthetic bacteria and to be the parent genus for the remaining free-living diazotrophs, the most recent of which is probably *Azotobacter*. In contrast IMSHENETSKII (332) regards *Clostridium pasteurianum* as the prototype diazotroph and feels that the numerous similarities between *Azotobacter* and certain blue-green algae indicate a much more direct evolutionary link between these organisms.

Evidence to support the evolutionary chronology outlined in this section is perforce circumstantial, but an examination of some characteristics of the $N_2$-fixing organisms shows a degree of consistency with the scheme described. The majority of $N_2$-fixing species are facultative, indicating they were present during the transition from a reducing to an oxidizing environment when the major development and proliferation of the ability to fix $N_2$ are suggested to have occurred. Most, possibly all, photosynthetic bacteria fix $N_2$; these organisms likely represented dominant species in the immediate preoxidative period by virtue of their ability to utilize solar energy. As such their high populations would favor a high incidence of adaptation to changing conditions, and it is reasonable that nitrogenase would develop initially among this group. The sensitivity of nitrogenase to $O_2$ suggests its origin in an essentially reducing environment. This is further indicated by the inability of the non-photosynthetic facultative bacteria to fix $N_2$ aerobically and by the greater $N_2$ fixation rates of photosynthetic bacteria growing under anaerobic *vs.* aerobic metabolism in only a few bacteria, such as *Azotobacter* and *Mycobacterium*, in the blue-green algae and in the higher plant symbioses. The $N_2$-fixing enzymes of these aerobic organisms are, nevertheless, susceptible to $O_2$ inhibition, and appear to function by virtue of unique protective mechanisms (p. 103).

The similarity of the nitrogenases and nitrogenase reaction among the diazotrophs suggests a common ancestry which appears difficult to reconcile with the diverse taxonomy of these organisms. The situation could have come about, however, if $N_2$ fixation had once been common among microorganisms, but subsequently was discarded by many of them. In the evolutionary chronology described here it is implicit that the ability to fix $N_2$ would have held great survival value in the post-denitrification period. Conceivably the majority of surviving species possessed this characteristic. However, as pools of fixed nitrogen became established, the need for nitrogenase would diminish and the enzyme might in fact be selected against. Much of this sort of genetic transition may well have occurred during the critical period of very rapid increase in atmospheric $O_2$ as green plants began their ascendancy. The pressures this change would exert on anaerobes would likely result in the jettisoning of all but the most essential enzyme systems.

However, since retention of this capacity would be of obvious value to both bacteria and plant, selection processes may well have favored a mechanism for "banking" nitrogenase DNA in the plant. Present data do not permit an adequate testing of this interesting concept; when it becomes possible to assign the genetic information for nitrogenase specifically to the host plant or to the bacteria, a more definitive proposal for the evaluation of obligatory symbioses can be made.

## II. Role of Diazotrophs in Global Nitrogen Economy

Developments within the past decade have done much to stimulate interest in the magnitude of $N_2$ fixation and the ecological significance of the diazotrophs. Partly responsible for this is the dramatic awakening of interest in all aspects of ecology, brought on by the mounting awareness of man's impact on his environment. Another major factor which has directed attention to the global role of nitrogen is the impending threat of world-wide food shortages in the wake of expanding populations. Next to water, nitrogen is the most frequently encountered limiting component in agriculture, and it is particularly critical in the production of increasingly scarce high protein foods. The need to bring more nitrogen into the food chain is evident.

Thus recent developments which are largely social in character have worked to focus attention on the global role of $N_2$ fixation. In so doing they have helped to expose the deficiency of basic data concerning this role. The most promising means to correct this deficiency appears to lie with a third recent development, quite unrelated to social considerations. This is the strictly technical advance of the $C_2H_2$ reduction assay for $N_2$ fixation (p. 10). Simply by providing the means to make the necessary types and numbers of analyses, this procedure has stimulated activity in the ecological and quantitative aspects of $N_2$ fixation. Its continued widespread application should lead to an increasingly accurate appreciation of the present and potential roles of $N_2$ fixation in the nitrogen economy of the earth.

### A. Nitrogen Cycle

In this section the nitrogen cycle will be described in qualitative and quantitative terms through consideration of five major nitrogen sinks and the intra- and intersink reactions which move nitrogen through the cycle. Like all other bio-essential elements, nitrogen is recycled on a global scale. The complex sequences of interacting reactions which comprise the nitrogen cycle encompass the metabolic activities of all living organisms and additionally include important nitrogen conversions in the abiological realm. The major sinks and nitrogen transfer and transformations involved in the global cycling of nitrogen are shown in Figure 3-1. Global movement of nitrogen is shown to occur through the operation of two major subcycles, designated the $N_2$ fixation-denitrification cycle and the precipitation-volatilization cycle. The former is essentially biological in character, and the latter abiological. In the atmosphere these cycles appear to function independently, but in the soil-water pool, the individual cycles lose identity as they interact and tend to equilibrate through common intermediates.

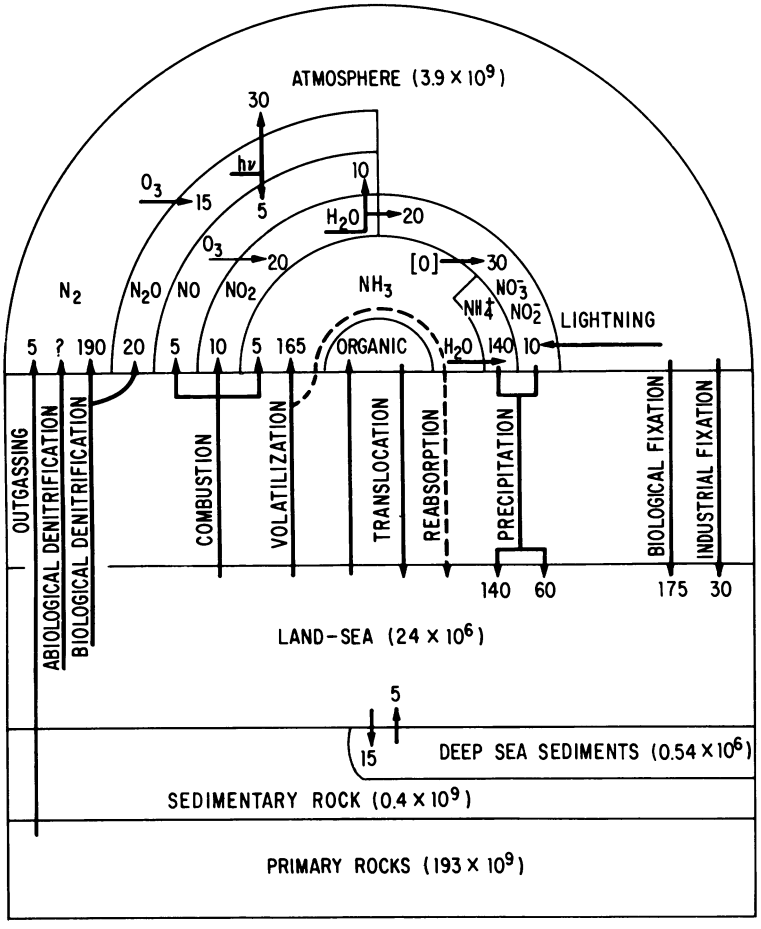


Fig. 3-1. Global cycling of nitrogen. Figures are millions of metric tons. Annual rates are shown for the nitrogen movements and conversions; nitrogen inventory values for the major sinks are shown in parantheses (Ref. 652, Tables 3-2, 3).

The rates for precipitation and for $N_2$ fixation were used as the key values to which others were equated. The basis for the precipitation rate is given below and the value for biological $N_2$ fixation is developed in Section II-B of this chapter. The annual rate of industrially fixed nitrogen was obtained from published figures (479). The data reviewed by ALLISON (11) indicate precipitation of about 0.7-0.9 g inorganic nitrogen/$m^2$ × yr, consisting of 70% $NH_4^+$-N and 30% $NO_3^-/NO_2^-$-N. The data of ERIKSSON (213) indicate about 0.4-0.8 g N/$m^2$ and those summarized by HENZELL and NORRIS (305) and KÖHLER (398) also fall generally in this range. Based on these sources a rate of about 0.7 g N/$m^2$ × yr was used for nitrogen in precipitation over land, and this is estimated to be 70% $NH_4^+$-N and 30% $NO_3^-/NO_2^-$-N. On a global basis this represents about 105 × $10^6$ metric tons/year for precipitation over land. A rate of 0.17 g N/$m^2$ × yr was estimated for nitrogen in precipitation at sea. This gives about 60 × $10^6$ metric tons on a global basis to agree with the value of 59 × $10^6$ metric tons estimated by MARTIN (452). Attributing 70% of this to $NH_4^+$ and 30% to $NO_3^-/NO_2^-$, as with precipitation over land, is consistent with the estimate by HORNE (319) that 0.03 g $NO_3^-$-N and 0.06-0.24 g $NH_4^+$-N/$m^2$ × yr are added to the sea in rain. The values of 0.7 and 0.17 g/$m^2$ × yr for nitrogen in wet precipitation over land and sea, respectively, are slightly more conservative than the estimates

A certain similarity is apparent in the pattern of the $N_2$ fixation-denitrification cycle and that of another major natural cycle, the carbon cycle. In both of these an atmospheric compound is fixed into the soil-water pool by energy-requiring reductive anabolic processes which produce reduced species that are then incorporated by biosynthesis into various cellular components and/or are subsequently returned to the atmosphere by catabolic reactions from which the catalyzing organism derives its energy. Another analogy, the similarity of the roles of $N_2$ in fixation and of $O_2$ in respiration, has often been noted, and the explanation of $N_2$ fixation as a form of respiration has been proposed (p. 42; Ref. 542).

*1. Nitrogen Sinks*

The earth's nitrogen reserves can be considered to reside in five major sinks: the primary rocks, the sedimentary rocks, the deep-sea sediment, the atmosphere and the soil-water pool (Fig. 3-1; Tables 3-2, 3). The primary rocks of the earth's crust hold about 12.5 g/N/$m^3$ for an estimated 97.8% of the global total[1], principally in the form of $N_2$. These rocks contribute almost nothing to the cycling of nitrogen, perhaps giving up about $5 \times 10^6$ metric tons $N_2$/yr via outgassing, and a considerably lesser amount through volcanic action. About 0.2% of all nitrogen is in sedimentary rocks, which sometimes contain relatively high levels of bound nitrogen. A recent survey of Wisconsin limestones indicated values for ammonia and nitrate frequently exceeding 2.5 g N/$m^3$, and it was suggested that the solution leached from these rocks may make significant contributions to the nitrogen content of groundwater (122). Nitrogen in deep-sea sediment accounts for only about $0.3 \times 10^{-4}$% of all nitrogen. It accumulates primarily by deposition of organic compounds, though sedimentation of clay-bound ammonia also occurs. Microbial recovery processes reconvert an estimated $5 \times 10^6$ metric tons/yr to soluble inorganic forms of nitrogen, but deposition probably exceeds recovery by about $10 \times 10^6$ metric tons/yr (452). In the atmosphere, which contains about 2% of global nitrogen, $N_2$ is by far the major species, but this sink also contains other nitrogen compounds which are important more for their mobilities and reactivities than for their abundance. Thus, small but significant steady-state levels of $N_2O$, NO, $NO_2$, $NH_3$, $NO_3^-$, $NO_2^-$ and $NH_4^+$ (the latter three as aerosols) occur there (Table 3-2). The next-to-smallest sink, designated the soil-water pool, is essentially the biosphere (Table 3-3). As in the atmosphere, $N_2$ is the predominant species, present here as a dissolved gas in the sea. The most reactive nitrogen compounds are the dissolved ionic species of the sea and the "available" or soluble $NO_3^-/NO_2^-$ and $NH_4^+$ nitrogen of the land. Less reactive spe-

[1] Based on 0.04 cc $N_2$/g igneous rock and a density of 2.5 g/cc (572).

Legend to Fig. 3-1 (continued). of 0.8 and 0.3 gN for these precipitations made by ROBINSON and ROBBINS (584). The estimate of $40 \times 10^6$ metric tons for annual dry deposition is based on JUNGE's estimate (356) that nitrogen in wet precipitation represents 80% of the total wet plus dry deposition.

The magnitudes of the lesser transformations are reasonably consistent with literature values, but the values for denitrification and $NH_3$ volatilization are forced in order to balance the cycle. The literature contains few values for comparison. While most of the transformations and transfers are discussed in the text, those which are strictly intra-atmospheric are not; these are listed in Table 3-4

Table 3-2. Atmospheric nitrogen inventory

| | Atm. mass[a] | Annual input[b] | Mean residence time |
|---|---|---|---|
| | (millions of metric tons) | | |
| $N_2$ | $3865 \times 10^6$ | 225 | $17 \times 10^6$ years |
| $N_2O$ | 1400 | 35 | 40 years |
| NO | 2 | 20 | 35 days |
| $NO_2$ | 4 | 30 | 36 days |
| $NO_3^-/NO_2^-$ | 0.2 | 60 | 1 day |
| $NH_4^+$ | 4 | 140 | 10 days |
| $NH_3$ | 27 | >170 | <58 days |

[a] from ROBINSON and ROBBINS, Refs. 584, 585
[b] from Fig. 3-1

Table 3-3. Soil-water pool nitrogen inventory

| Component | Sea | Land | Reference |
|---|---|---|---|
| | (millions of metric tons) | | |
| Soluble nitrogen | | 1,000 | a |
| $NO_3^-$ | 650,000 | | b |
| $NO_2^-$ | 5,000 | | 452 |
| $NH_4^+$ | 5,000 | | 452 |
| Insoluble inorganic | | 100,000 | c |
| Coal nitrogen | | 100,000 | 190 |
| Organic nitrogen | 650,000 | 550,000 | d |
| Animals | 3,000 | 1,000 | e |
| Plants | 1,000 | 10,000 | e |
| | 1,300,000 | 750,000 | |
| $N_2$ dissolved in sea | 22,000,000 | | 452 |

[a] Estimated at 1% of insoluble inorganic nitrogen.
[b] Average of 570,000 (452) and $700,000 \times 10^6$ metric tons (574).
[c] Value given as land inorganic nitrogen in Ref. 162, rounded off from $140,000 \times 10^6$ metric tons; primarily clay-bound ammonia, but includes such sources as nitrate deposits, estimated at about $100 \times 10^6$ metric tons.
[d] Sea value is average of 700,000 (190), 900,000 (162) and $340,000 \times 10^6$ metric tons (452); land value based on the ratio of land:sea organic nitrogen given in Ref. 574.
[e] Evaluated from the estimates of Ref. 162 and 190.

cies include organic nitrogen and the so-called "fixed" ammonia, i.e., ammonia complexed to clay and in weak equilibrium with the soluble $NH_4^+$.

*2. Nitrogen Transformations and Transfers*

The nitrogen conversions and transfers shown in Figure 3-1 are presumed to maintain the global nitrogen inventory in a steady-state condition, but it is difficult to establish the accuracy of such a scheme because of the uncertainty in assessing many of the actual

pool sizes and accretion/depletion rates. The most reliable values are those given for sizes of the larger atmospheric pools, the amount of $N_2$ dissolved in the sea and the amount of industrially fixed $N_2$. Most other values represent best estimates frequently derived from conflicting literature reports.

The transformations and transfers which comprise the nitrogen cycle presented here represent the authors' interpretation of the relevant literature. Unquestionably, new data will require that the cycle be modified both quantitatively and qualitatively. The current portrayal does in fact contain the seeds for such change by indicating possible roles for such phenomena as NO volatilization and the scavenging of various atmospheric species by plants and soil. Evidence for such mechanisms is only now entering the literature, but like other, still undiscovered, mechanisms they may prove to be of great importance in the cycling of nitrogen.

a) Soil-Water Pool

The entry of nitrogen into the soil-water pool from the atmosphere occurs by two main routes: $N_2$ fixation and aerosol nitrogen precipitation. The amount of $N_2$ fixed industrially by the Haber-Bosch method is obtainable from production figures and currently represents an input of about $30 \times 10^6$ metric tons per year for both agricultural and industrial use (479). This rate has increased dramatically in modern times and is anticipated to reach about $44 \times 10^6$ metric tons/yr by 1975 (15). The annual rate of biological $N_2$ fixation is more difficult to assess, and the basis for an estimated rate of $175 \times 10^6$ metric tons is considered in detail in a subsequent section. The amount of $200 \times 10^6$ metric tons for nitrogen in annual precipitation is within the range reported from numerous sources, and the distribution among $NH_4^+$ and $NO_3^-/NO_2^-$ in precipitation reflects the approximate balance which frequently has been observed, though wide local and seasonal variations exist in this distribution (see legend to Fig. 3-1). Hitherto unappreciated nitrogen accretion mechanisms may involve the recently described ability of plants to scavenge NO and $NO_2$ from the atmosphere (2, 40, 326, 561). At present there is insufficient basis for evaluating annual rates for these foliar absorption mechanisms. The reabsorption of volatilized $NH_3$ and the resettlement of dust-bound organic nitrogen are considered below as translocations within the pool.

The soil-water pool is clearly the dynamic center of the nitrogen cycle, and just as clearly this is a consequence of the magnitude and variety of nitrogen conversions mediated by living organisms. The interrelationships among these conversions and also the abiological reactions of this pool are outlined in Figure 3-2. While all reactions shown are important in maintaining a relatively stable steady-state condition in the pool, the processes of $N_2$ fixation, nitrification and denitrification must be considered particularly crucial to the global cycling of nitrogen.

An examination of the biological nitrogen reactions shows that two main types of activity most directly affect the cyclic movement of nitrogen. These may be called synthesis-related reactions and energy-related reactions, and they are considered in detail below. Additionally, intercellular nitrogen conversions and hydrolytic reactions associated with catabolism occur, but the former are of little relevance in the present context, and the latter are of peripheral significance, serving mainly to provide an intrapool flux of simple nitrogen com-

pounds, such as ammonia, amino acids, urea, uric acid and bile salts; these may be broken down further, and/or they may enter various organic and inorganic nitrogen pools.

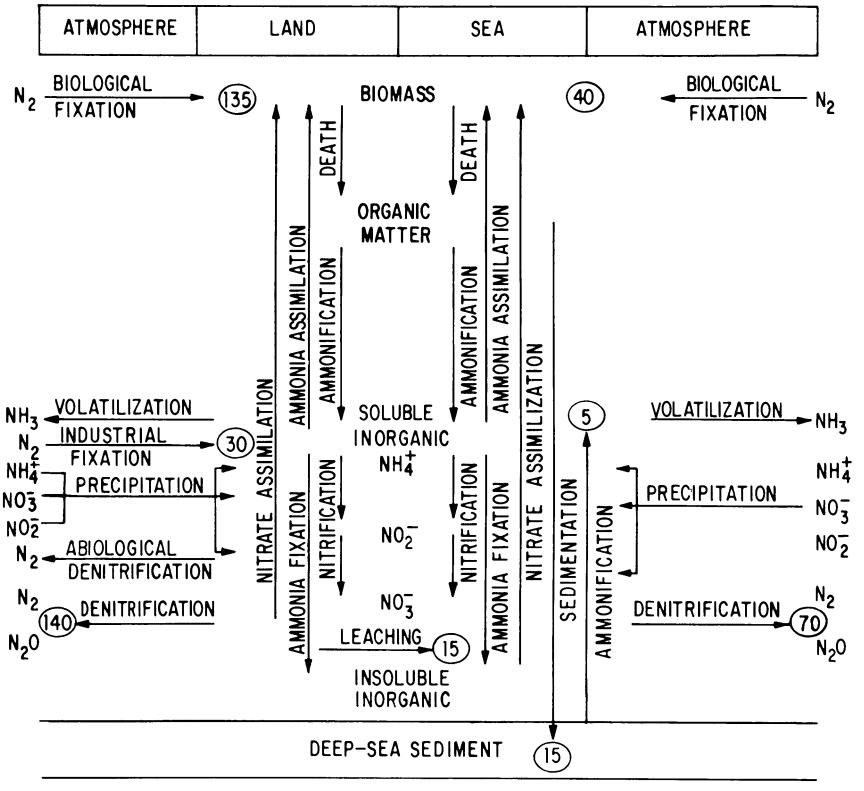


Fig. 3-2. Nitrogen interconversion in soil-water pool. Figures are millions of metric tons per year

Included in the synthesis-related nitrogen conversions are $N_2$ fixation and the reactions which incorporate ammonia, nitrite and nitrate into living cells. Of these, only $N_2$ fixation brings nitrogen into the soil-water pool; the others simply utilize nitrogen compounds available in the pool. Almost without exception microorganisms and plants can utilize ammonia or nitrate and can usually assimilate both. The assimilation of nitrite is much less extensive because of its toxicity; most is probably converted to nitrate. These oxidized forms are reduced to ammonia for incorporation into cell material. The enzymic reactions responsible for this and for ammonia incorporation, as well as the reconversion of cellular nitrogen are well documented (e.g. Refs. 5, 6, 517) and will not be discussed here.

Energy-related nitrogen conversions which are critical for nitrogen cycling include nitrification and denitrification (p. 40). In nitrification, which is aerobic, nitrogen functions as the reductant and undergoes a valency change from -3 in ammonia to +5 in nitrate:

$$NH_4^+ \xrightarrow{[O]} NO_2^- \xrightarrow{[O]} NO_3^-$$

In denitrification, which is anaerobic, nitrogen functions as the oxidant and gaseous products are formed. The valency change here is from +5 in nitrate to +1 in $N_2O$ or to 0 in $N_2$:

$$NO_3^- \longrightarrow NO_2^- \longrightarrow N_2O,\ N_2$$

The denitrification reaction sequence is the special case of dissimilatory nitrate-utilization in which the process goes beyond the formation of nitrite (517). Both nitrate and nitrite can serve as the initial substrate. Nitrification and denitrification are successive links in the routing of nitrogen through and back out of the soil-water pool. For the organisms which carry on these reactions, nitrification and denitrification are normally the essential energy yielding processes.

The nitrification reactions appear to be major metabolic functions only of the autotrophic bacteria which comprise the family Nitrobacteriaceae (order Pseudomonadales). Seven species are listed in BERGEY's Manual (54), though recently two additional genera, *Nitrospira* and *Nitrococcus*, were proposed for marine isolates capable of oxidizing nitrite to nitrate (704). The nitrifying genera fall into two groups, those which oxidize ammonia to nitrite and those which oxidize nitrite to nitrate; curiously, none appears capable of performing both reactions. The genus *Nitrosomonas* appears to be the most widespread of the former type, and *Nitrobacter* the most prevalent nitrite oxidizer. The characteristic oxidations can be written as follows (5, 162):

$$NH_4^+ + 3/2\ O_2 \xrightarrow{Nitrosomonas} NO_2^- + 2\,H^+ + H_2O \quad (\Delta F = -65 \text{ kcal/mole } NH_4^+)$$

$$NO_2^- + 1/2\ O_2 \xrightarrow{Nitrobacter} NO_3^- \quad (\Delta F = -18 \text{ kcal/mole } NO_2^-).$$

The energetics of these reactions have been examined in detail by ALEEM (5).

Other microorganisms, including certain bacteria, molds and fungi, are capable of a limited oxidation of nitrogen compounds (6), but their over-all contribution to nitrification is believed to be small; attempts to demonstrate nitrification by heterotrophs in the sea have been unsuccessful (118). In soil, free ammonia is rapidly oxidized by the endogenous population of nitrifying bacteria. This oxidation is of considerable agricultural and ecological importance, since fertilizer ammonia is thus often rapidly converted to nitrate, in which form nitrogen is more likely to be lost from the soil either by leaching or denitrification. The latter process proceeds vigorously in most soils under anaerobic conditions, and since nitrification consumes $O_2$, the nitrifiers may encourage denitrification both by supplying the substrate and by providing a favorable environment for reaction.

Denitrification is carried out by a more diverse group of bacteria than is the case with nitrification. The process is generally considered to take place only under anaerobic conditions, but exceptions to this have been reported (see below). Substrate and product specifities are not well defined among the denitrifiers, but it appears that some can utilize only nitrate or nitrite, while others can utilize both. It is not clear whether species specifictiy or environmental factors determine between $N_2$ or $N_2O$ in the products. Two different types of denitrifying agents may be distinguished. The first includes non-spore formers from such genera as *Pseudomonas*, *Micrococcus* and *Achromobacter*; the second group consists of certain spore-forming facultative anaerobic species of *Bacillus*.

The non-spore forming denitrifiers are referred to as strict aerobes, but this is a misnomer which is particularly misleading in the present context, since their ability to denitrify uniquely permits them to function in the absence of $O_2$. In contrast to facultative aerobes, which simply convert to a fermentative metabolism under anaerobic conditions, these denitrifying agents can use nitrite or nitrate as a substitue for $O_2$. With these compounds functioning as terminal electron acceptors for their cytochrome systems, these organisms continue to carry out the complete combustion of carbon compounds to $CO_2$ and water. This metabolic mode permits significant energy yields, as indicated below (162).

$C_6H_{12}O_6 + 6\ NO_3^- \rightarrow 6\ CO_2 + 3\ H_2O + 6\ OH^- + 3\ N_2O$ ($\Delta F^o$ = -545 kcal/mole glucose)

$5\ C_6H_{12}O_6 + 24\ NO_3^- \rightarrow 30\ CO_2 + 18\ H_2O + 24\ OH^- + 12\ N_2$ ($\Delta F^o$ = -570 kcal/mole glucose).

The spore-forming denitrifiers can couple nitrate or nitrite only into their fermentative metabolism, and accordingly the energy yields are usually relatively low. The utilization of nitrate is not restricted to a carbon-based energy metabolism, as the following reaction of *Thiobacillus* demonstrates (609).

$5\ Na_2S_2O_3 + 8\ KNO_3 + 2\ NaHCO_3 \rightarrow$
$6\ Na_2SO_4 + 4\ K_2SO_4 + 2\ CO_2 + H_2O + 4\ N_2$ ($\Delta F^o$ = -179 kcal/mole $Na_2S_2O_3$).

Very little data exist on the extent and rates of denitrification on a global scale, but the process has received considerable attention on crop lands, where it causes substantial nitrogen losses. Denitrification losses of 10-30% of added fertilizer nitrogen are considered normal (81, 403, 748). The ubiquity of the denitrifying agents in soil suggests that the process will occur whenever substrate availability and environmental conditions permit. More recently it has become evident that appreciable denitrification occurs in aquatic areas as well (80, 249, 363, 381, 746). A consideration of great significance in evaluating denitrification rates *in situ* is the effect of $O_2$. Though it is generally assumed that anaerobic or micro-aerophyllic conditions are essential for denitrification, this point is not yet well established. For example, substantial nitrite reduction has been reported for *Achromobacter* (628) and *Micrococcus* (364) under aerobic conditions. To the extent that aerobic denitrification occurs in nature, denitrification rates have probably been underestimated. In Figures 3-1, 2 the rate for annual denitrification is a forced figure, developed to approximately balance the rate of $N_2$ fixation. The product balance shown for denitrification gives an $N_2$:$N_2O$ ratio of almost 10; this is consistent with existing data and provides an amount of $N_2O$ equivalent to the amount calculated to be consumed annually in scavenging reactions in the atmosphere (22).

Except for dissolved $N_2$, most of the nitrogen in the soil-water pool is associated with organic matter. This nitrogen is primarily of biological origin, though a significant amount of the organic nitrogen in soil results from abiological "ammonia fixation" in which $NH_3$ complexes with soil organic matter, yielding varied and largely unidentified products (521). Additional ammonia fixation, involving the complexation of $NH_4^+$ with clay particles, ties up more ammonia, so that the amount of free, or available $NH_4^+$ is small relative to bound forms. Nitrogen is released from organic matter by bacterial action termed "mineralization", a process which releases perhaps 1-2% of organic nitrogen as $NH_4^+$ per year, while fixed ammonia is liberated from clay in response to equilibrium demands. Additional nitrogen is released

by abiological denitrification (81). Though the reactions involved are not well understood, in the case of organic matter a Van Slyke-type reaction may occur resulting in the loss of nitrogen to the atmosphere:

$$RNH_2 + HNO_2 \rightarrow ROH + H_2O + N_2$$

Volatilization of $NH_3$ formed in the equilibirium $NH_4^+ + H_2O \rightleftarrows NH_3 + H_3O^+$ results in a substantial loss of nitrogen from the soil water pool to the atmosphere. While much of this $NH_3$ is reabsorbed without undergoing change in the atmosphere (see below), reactivity in the atmosphere is indicated for about $170 \times 10^6$ metric tons/yr (Fig. 3-1). Like the denitrification value, this rate is a forced figure calculated in this case to counterbalance nitrogen recycled via precipitation. Other transformations and transfers which bring nitrogen into the atmosphere include the combustion of fossil fuels to yield ammonia, NO, and $NO_2$ and the outgassing of primary rock. The values shown here are based on current literature estimates (584, 585). Although the addition of nitrogen oxides to the atmosphere by combustion is relatively small, this source has gained prominence because of its contribution to pollution. An additional mechanism, the volatilization of NO, has recently been reported (651); this is indicated to occur abiologically under aerobic conditions, but at present there is no basis for assessing its possible global significance.

In addition to the numerous chemical conversions which involve nitrogen in the soil-water pool, two major physical transfer mechanisms also occur: 1. the volatilization and reabsorption of ammonia referred to above, and 2. the pick-up and resettlement of dust-bound organic nitrogen. Though these movements occur through the atmosphere, they are more practically considered as intrapool effects involving translocations only in the soil-water pool. Total ammonia volatilization may involve as much as $682 \times 10^6$ metric tons N/yr, according to ROBINSON and ROBBINS (585), but this is more than 4.5 g/m$^2$ [2] of land, and seems improbably high. Most is probably reabsorbed close to its point of origin, making the over-all effect of this type of translocation small in spite of the vast amounts presumably involved. However, its significance for nitrogen cycling may be greater than supposed, if the recently reported foliar absorption of ammonia occurs on a significant scale (326, 561). The sea, being slightly alkaline, may generate a portion of the volatile $NH_3$, but by far the bulk must be considered to originate on land, as must most reabsorption. The airborne movement of organic nitrogen may also involve substantial amounts. A survey by ERIKSSON (213) indicated as much as 0.4-0.6 g organic N/m$^2$ to be deposited annually; based on total land area and 0.5 g/m$^2$, a value of about $75 \times 10^6$ metric tons of organic nitrogen per year for the earth can be estimated. In contrast to ammonia translocation these movements may have more far-reaching distributional effects and result in a net movement of nitrogen from land to sea. The translocations of ammonia and of organic nitrogen both tend to equalize the distribution of nitrogen in the biosphere by drawing on relatively nitrogen-rich areas and donating to relatively nitrogen-poor areas. For example, areas of concentrated livestock production, such as feedlots, produce large quantities of ammonia, and the redistribution of this ammonia can constitute the major nitrogen input in surrounding areas. Such redistributions do not represent cycling in the chemical sense, but they unquestionably influence chemical cycling by the large-scale

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[2] The following conversions may be useful: 1 g/m$^2$ = 10 kg/hectare (ha) = 8.93 lb/acre.

movement of substrates and products to and from areas more or less conducive to reactivity.

b) Atmosphere

Atmospheric nitrogen reactions are shown to be wholly abiological (Table 3-4), though a recent report (541) suggests that appreciable biological activity may take place in this sink. Table 3-2 shows the mean life times and turnover rates of atmospheric nitrogen compounds. In passing, it is of interest to contrast the mean life time of 17 million years for aerial $N_2$ with a recent widely distributed statement, "For example at least six types of bacteria in soil and water are absolutely essential to keep nitrogen circulating from air into organic material, then back to air again. If any of the bacteria stopped working, nitrogen in the atmosphere would be depleted or possibly replaced by ammonia" (133); while this statement is true

Table 3-4. Reactions in the atmosphere

| | | Reference |
|---|---|---|
| Ozonization of $N_2$ | $N_2 + O_3 \rightarrow N_2O + O_2$ | 27, 251 |
| Lightning | e.g. $O^+ + N_2 \rightarrow NO^+ + N$<br>$NO^+ + H_2O \rightarrow 2H^+ + NO_2^-$ | 230, 325 |
| Photolysis of $N_2O$ | $N_2O \rightarrow N_2 + O$<br>$N_2O \rightarrow NO + N$ | 27, 584, 585 |
| Ozonization of NO | $NO + O_3 \rightarrow NO_2 + O_2$ | 584, 585 |
| Oxidation of NO | $2NO + O_2 \rightarrow 2NO_2$ | 420 |
| Hydration of $NO_2$ | $3NO_2 + H_2O \rightarrow 2HNO_3 + NO$ | 584, 585 |
| Oxidation of $NH_3$ | $NH_3 + [O] \rightarrow^{(?)} \rightarrow NO_3^-$ | 325, 585, 652 |
| Hydration of $NH_3$ | $NH_3 + H_2O \rightarrow NH_4^+ + OH^-$ | 584 |
| Acid aerosol neutralization of $NH_3$ | $NH_3 + HNO_3 \rightarrow NH_4NO_3$<br>$2NH_3 + H_2SO_4 \rightarrow (NH_4)_2SO_4$ | 584<br>584 |

it ignores the relatively long time factor. In contrast to the soil-water pool, little intrapool cycling appears to take place in the atmosphere. Most atmospheric $NH_3$ is rapidly converted to aerosol $NH_4^+$ by hydration followed by neutralization or by direct $NH_3$-acid aerosol neutralization to give $(NH_4)_2SO_4$ or $NH_4NO_3$ (584). The remaining $NH_3$ is thought to be scavenged by oxidation to nitrate; the reaction(s) responsible has not been established, though photochemical oxidation has been suggested (325, 584, 652).

The extent to which the other indicated atmospheric reactions listed in Table 3-4 actually take place and the completeness of the listing are difficult to document. For example, the amount of $N_2$ fixed via oxidation by lightning is shown to be about 20% of the nitrogen precipitated as $NO_3^-/NO_2^-$, in agreement with previous estimates (325) for the fractional contribution of lightning, but with little basis in verifiable experimentation.

## Concluding Remarks

It can be concluded that the nitrogen cycle is reasonably well established in qualitative terms of sinks, transformations and transfers, but still ill-defined in quantitative terms. From an ecological viewpoint quantitative data are essential, and obtaining this information remains a challenge for the coming decade. Clearly new methodology and extensive *in situ* analyses are required if such processes as ammonia volatilization and denitrification are to be properly evaluated. The relative impact of industrial and biological $N_2$ fixation on the land requires further clarification, as does the effect of these alternative nitrogen sources on nitrate concentrations of surface waters. The availability of such definitive data will permit the planning of rational, ecologically-acceptable programs for the addition of essential nitrogen to the food chain.

## B. Amount of $N_2$ Fixed

From the increasing number of reports of $N_2$ fixation in the biosphere it is becoming apparent that $N_2$ fixation is both more intense and more widespread than had previously been supposed. Some of the mounting evidence leading to this conclusion can be summarized as follows:

1. The blue-green algae are becoming recognized as highly significant factors in global $N_2$ fixation (656, 658, p. 23). Dinitrogen fixation by various species has been observed in numerous locales not previously investigated, including many marine environments. A broader active temperature range for these agents is now indicated by documentation of extreme cases of blue-green algae growing in hot springs and at just-above-freezing. Furthermore, it is now established that $N_2$ fixation among the blue-green algae is not limited to heterocystous, filamentous species as had been supposed, but extends to to some non-filamentous and non-heterocystous species as well.

2. Many aerobic $N_2$-fixing species, including both bacteria (306, 467) and blue-green algae (667), have been shown to fix more effectively at $O_2$ levels below 0.2 atmospheres (p. 103), as is frequently encountered in subsoil and marine environments. In fact, the long-held belief that blue-green algae are obligate aerobes has been shown to be incorrect, and some species grow even better under reducing conditions than in air (see discussion in 306); the prevalence of $N_2$-fixing blue-green algae in many successful symbioses, such as lichens, may result in part from the influence of the non-algal partner in maintaining the algae in partial anaerobiosis.

3. The wide range and prevalence of associative symbioses are coming more sharply into focus as documentation of phylloplane and rhizosphere $N_2$-fixing associations continues to enter the literature (p. 26). Clearly these collaborations have been little appreciated in the past, yet preliminary assessment indicates that they exert a significant impact on $N_2$ fixation rates associated with a great variety of ecosystems.

4. Consistent reports from several sources indicate that $N_2$ fixation may occur in the digestive tracts of ruminants and possibly even humans (p. 28); while not highly significant in terms of total $N_2$ fixed (about 1000 metric tons/yr based on published rates), this finding obviously represents yet another previously overlooked factor in biospheric $N_2$ fixation.

These reports describe a much more complex and extensive involvement of free-living diazotrophs in the biosphere than was previously supposed. They support the suggestion of STEWART (654) that the contribution of these agents has been underestimated in the past and should be re-evaluated. The concept that $N_2$ fixation from this source was relatively unimpressive has its origins in the long-held belief that *Azotobacter* and *Clostridium* were the only (or at least the only effective) free-living diazotrophs, and that even they were seldom very effective. For lack of better methods, free-living $N_2$ fixation in the soil was often equated with easily obtained *Azotobacter* counts (482); analyses of actual $N_2$ fixed based on nitrogen determinations varied widely and were generally discounted because of the large margins of error. Even with the advent of more sophisticated methods, the failure to appreciate such important factors as $pO_2$ and interorganismal associations must often have resulted in minimal responses. On theoretical grounds the argument is frequently advanced that the amount of energy required for $N_2$ fixation (based on pure culture analyses) precluded any substantial degree of free-living $N_2$ fixation in the field; while it is true that additions of organic matter generally stimulate $N_2$ fixation in the soil (229, 285), MOORE has pointed out (482) that this concept makes little sense where photosynthetic diazotrophs are concerned, and in view of the widespread occurrence of associations between diazotrophs and other organisms, particularly those involving higher plants, the argument may have little relevance to many biospheric conditions.

### *1. $N_2$ Fixation Rates in Various Ecosystems*

From the above considerations and from the information elaborated in Table 3-5, a value of $175 \times 10^6$ metric tons was developed for the estimated annual amount of biologically fixed N. The $N_2$ fixation rates are based on a broad range of literature reports for the various ecosystems; representative values are summarized in Table 3-6. Because of their agricultural significance the analytical data on legumes are much more comprehensive than those on any other major $N_2$-fixing group. Analysis by the Kjeldahl method can be generally considered reliable with these crops, since the amount of $N_2$ fixed is large relative to background, and additionally it is retained in the plant which is readily isolated for analysis; the contribution made by soil nitrogen can be evaluated from soil nitrogen assays. $C_2H_2$ reduction assays, while still less numerous than Kjeldahl nitrogen analyses, are in good agreement with Kjeldahl results (275).

The rate estimates given for areas other than land in cropped legumes are more uncertain. The Kjeldahl method has been applied less successfully in assessing $N_2$ fixation in those areas where fixed $N_2$ seldom accumulates in readily isolated plants and in amounts well above endogenous levels. The contributions made by free-living diazotrophs have thus at times been estimated from bacteria counts or from fixation rates observed with isolates under laboratory conditions, and these may bear little relationship to the actual intensity of $N_2$ fixation occurring *in situ*. Many difficulties in field evaluation of $N_2$ fixation are overcome by the $C_2H_2$ assay, which is readily applied to field conditions.

The value for the annual rate of $N_2$ fixation developed in this section is higher than most previous estimates, due primarily to the influence of the more recent reports, as alluded to previously. The basis of the values for each of the ecosystems listed in Table 3-5 is discussed below.

Table 3-5. $N_2$ Fixation in the biosphere

| Land use[a] | ha × $10^{-6}$ | | | kg $N_2$ fixed per ha × yr[b] | Metric tons per yr (×$10^{-6}$) |
|---|---|---|---|---|---|
| Agricultural | | | 4400 | | |
| Arable under crop | | 1400 | | | |
| Legumes | 250 | | | 140 | 35 |
| Pulses | 63 | | | | |
| Soybeans | 34 | | | | |
| Groundnuts | 18 | | | | |
| Other | 135 | | | | |
| Non-legumes | 1150 | | | | |
| Rice | 135 | | | 30 | 4 |
| Other | 1015 | | | 5 | 5 |
| Permanent meadows, grasslands | | 3000 | | 15 | 45 |
| Forest and woodland | | | 4100 | 10 | 40 |
| Unused | | | 4900 | 2 | 10 |
| Ice covered | | | 1500 | 0 | 0 |
| Total land | | | 14,900 | | 139 |
| Sea | | | 36,100 | 1 | 36 |
| Total | | | 51,000 | | 175 |

[a] Land-use figures are from Ref. 211. Arable land includes land under crops, land temporarily fallow, temporary meadows for mowing or pasture, land under garden, fruit trees, vines, shrubs and rubber plantations. Land in legumes is estimated at 250 × $10^6$ ha or 18% of the arable land, somewhat below the estimate of 20-25% of the cultivated area made by MISHUSTIN and SHIL'NIKOVA (476) for countries with a highly developed agriculture. Permanent meadows and grasslands refers to land under herbaceous forage crops other than rotation grasses and clovers. Forested land includes land under natural or planted stands of trees. Unused land includes some potentially productive land, but primarily consists of wasteland and built-on areas.

[b] See footnote[2], p. 51.

Table 3-6. Reported rates of $N_2$ fixation by specific systems

| $N_2$-fixing system | kg $N_2$ fixed/ha × yr[a] | Reference[b] |
|---|---|---|
| Soybean | 94 | 654 |
| Soybean | 84 | 274 |
| Soybean | 57 | 210 |
| Soybean, groundnut | 91 | 525 |
| Pulses | 50-60 | 476 |
| Peas, lentils, vetches | 85 | 525 |
| Cowpeas | 84 | 210 |
| Groundnuts | 47 | 210 |
| Cereal legumes | 50 | 525 |
| Clovers | 105-220 | 210 |
| Clovers | 137 | 525 |
| Clovers | 150-160 | 476 |
| Clovers | 104-149 | 654 |
| Lucerne | 128 | 525 |
| Lucerne | 208 | 210 |
| Lucerne | 158 | 654 |

Table 3-6. (continued)

| $N_2$-fixing system | kg $N_2$ fixed/ha × yr[a] | Reference[b] |
|---|---|---|
| Lucerne | 300 | 476 |
| Lupin | 150 | 476 |
| Lupin | 169 | 210 |
| Mixed legumes | 125 | 654 |
| Pasture legumes | 118 | 525 |
| Rice paddy | 10-55 | 446 |
| Rice paddy | 27 | 703 |
| Rice paddy | 45 | 525 |
| Wheat field soil | 4 | 668 |
| Corn field soil | 90 | 627 |
| Soil, cultivated layer, USSR | 5 | 476 |
| Soil, Nile valley | 15 | 344 |
| Fallow soil, Calif. | 3.5 | 668 |
| Under native vegetation | 2 | 668 |
| Under grasses, iris | 45-66 | 245 |
| Soil, Quebec (anaerobic) | to 73 | 123 |
| Under mustard | 24 | 634 |
| Soil, Canadian | 2-200 | 392 |
| Soil, algal film | to 6 | 616 |
| Under rye grass | 55 | 545 |
| Natural grassland | 1-2 | 548 |
| Irrigated lawn | 5 | 668 |
| Grass | 112-145 | 481 |
| Sand dune vegetation | 0.1 | 4 |
| Beech forest | 0.4 | 323 |
| Coniferous forest | 50 | 576 |
| Pine forest | 0-23 | 355 |
| Pine forest | 13-39 | 322 |
| Soil under Douglas fir | 13-38 | 353 |
| Douglas fir, phylloplane | 7-23 | 353 |
| *Alnus* | 156 | 132 |
| *Alnus* | 85 | 697 |
| *Alnus* | 150 | 149 |
| *Alnus* | 56 | 4 |
| *Hippophaë* | 179 | 666 |
| *Hippophaë* | 2-15 | 4 |
| *Acacia* | 270 | 526 |
| *Casuarina* | 58 | 187 |
| *Ceanothus* | 60 | 165 |
| *Myrica* | 9 | 66 |
| *Gunnera-Nostoc* | to 72 | 624 |
| Desert crust, Arizona | 41 | 458 |
| Desert crust, Australia | 2-3 | 682 |
| Lichens, mountains | 39-84 | 253 |
| Lake, Wisconsin | 4 | 79 |
| Lake, Wisconsin | 2.4 | 665 |
| Lake, England | 0.4-3 | 317 |
| Lake, England | 8 | 235 |
| Lake, Tropical | 44 | 318 |
| Marine angiosperms, rhizosphere | 700 | 547 |

[a] The values for legume $N_2$ fixation are means and averages of many literature values, as indicated in the references.

[b] Additional listings can be found in Refs. 32, 131, 285, 348, 482, 654, 655, 658.

a) Cultivated Land

In agriculture an estimated 140 × $10^6$ metric tons[3] of nitrogen are removed from the land each year. Since the annual addition of fertilizer nitrogen is only about 25 × $10^6$ metric tons, of which not more than 50% may be utilized (10), manuring and biological $N_2$ fixation must supply well over 100 × $10^6$ metric tons/yr. In cultivated areas $N_2$ fixation is effected primarily by legumes, and these plants play an important role in the economy of almost every major agricultural area. Depending on the nitrogen content of the soil, these crops can utilize $N_2$ for 80% or more of their nitrogen (191, 441). In countries with a highly developed agriculture, for example, up to 25% of the area under cultivation is planted with legumes (476). In England legumes are estimated to supply 42% of annual nitrogen needs, with the balance from fertilizer (27%) and manuring (31%) (476). Legume fields are highly intensive areas of $N_2$ fixation, characterized by $N_2$ fixation rates considerably above the rates associated with other $N_2$-fixing sources. A considerable range in values of $N_2$ fixed is apparent (Table 3-6), and the value of 140kg/ha used in Table 3-5 may be slightly conservative, but is in agreement with a recently estimated weighted average (654).

Dinitrogen fixation by blue-green algae in cultivated land is significant probably only in rice culture (333, 393, 474, 505, 735), though crusts of blue-green algae have been observed in sugarcane fields (482) and other non-paddy fields (482, 616, 660). In rice paddies the annual average fixation rate is estimated here at 30 kg/ha. Recent reports (333, 393, 446, 745) show that $N_2$-fixing bacteria contribute to this total, but the bulk of $N_2$ fixation is probably due to algal action. Attempts to expand this by seeding paddies with blue-green algae have produced some encouraging results, with increases in rice yield of up to 20% reported (694, 703). Benefits from algal seeding were also indicated for lettuce and pepper culture (694).

Dinitrogen fixation by free-living bacteria in cultivated land is probably widespread, but generally of low intensity with considerable variation in response to changing environmental conditions (668). Both aerobic and anaerobic diazotrophs are found; anaerobic activity, usually attributable to *Clostridium*, is normally greater (392, 549), though this is not always the case (26). Specific rhizosphere and phylloplane associations may be important here in supporting $N_2$-fixing activity. Such associations have been demonstrated in the laboratory, but not *in situ*, with sugarcane (183), rice (188, 580) and bahai grass (184) and are indicated for wheat (668). High levels of $N_2$ fixation appear to be maintained by free-living diazotrophs in certain areas such as parts of the chernozem zone in the USSR where good crop yields have been reported for over 300 years without benefit of nitrogen fertilizer (476). Attempts to improve agricultural land and increase crop yields by sowing $N_2$-fixing bacteria, particularly *Azotobacter*, however, have met with only limited success (33, 260, 475, 591).

b) Forests and Grasslands

All categories of $N_2$-fixing agents are well represented in established uncultivated productive areas. Since these organisms comprise the pri-

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[3] Based on 1961 estimate of KOLAR and GREENLAND (399) of 100-110 × $10^6$ metric tons times a factor $\frac{155}{120}$ estimated from p. 27, Ref. 211, to account for production increase, 1961 to 1970.

mary means for replacing lost nitrogen, it is not unreasonable that they have been accommodated in the evolution of successful ecosystems. The magnitude of nitrogen loss from natural systems can be significant. HIROSE, SONG and MONSI (309) estimate the annual turnover of nitrogen in productive ecosystems to be 10-50 g $N/m^2$ × yr. In an undisturbed New England forest, loss through nitrate leaching alone was measured at 6 g $N/m^2$ × yr, and since the area experienced a net annual nitrogen increase estimated at 0.2 g/$m^2$, well in excess of 6 g $N/m^2$ must have been fixed (73). Nitrogen accretions of up to 5 g/$m^2$ × yr (576) may frequently occur in coniferous forests, and a recent study indicates that roughly half of this may occur via $N_2$ fixation by free-living microorganisms in the phylloplane (353). Significant contributions are also made in these areas by rhizosphere microorganisms (84, 576, 577, 578, 634) and by nodulated non-legumes such as *Comptonia* and *Purshia* which occur commonly in North American coniferous forests (466, 705). An analysis of two Japanese coniferous forests indicated that organisms other than *Azotobacter*, *Beijerinckia* and *Clostridium* accounted for the observed soil $N_2$ fixation (674).

Grasslands and meadows contain a wide range of leguminous species; in most areas these occur naturally [27 species were detected in western Dakota grasslands (131)], but the introduction of commercially important legumes in large-scale pasture improvement programs has met with considerable success, particularly in southern Australia (190). Free-living diazotrophs are also prevalent in grasslands, where the bacteria often appear to be closely associated with the roots or aerial portions of the grasses (41, 186, 549, 591, 668). A dependency of $N_2$ fixation on light in bahai grass *(Paspalum)* (361) suggests that the high concentrations of *Azotobacter* associated with the roots of these plants depend on photosynthate from the grass. Similarly, the number of *Azotobacter* in the rhizosphere is reported to be light independent (185); however, others have found a low level of $N_2$ fixation in turf to be independent of light (660). In an extensive California study involving monthly analyses, annual $N_2$ fixation rates were found to be roughly comparable in fallow soil, an irrigated lawn, under native vegetation and in a wheat field, ranging from 0.2-0.5 g $N_2$ fixed/$m^2$ × yr (668); however, all analyses were performed aerobically and significant anaerobic or micro-aerophyllic $N_2$ fixation may have been overlooked.

c) Unused Land

Included in this category (Table 3-5) are vast areas of marginal land such as desert, tundra and barren lands where survival is difficult and relatively few forms of life persist. Among those that can, survive, numerous $N_2$-fixing species are encountered; in nitrogen-deficient areas the diazotrophs have a decided competitive advantage. Blue-green algae in particular are hardy and able to develop at low levels of inorganic nutrients (see discussion in 234). Numerous species of blue-green algae are characteristic of marginal areas, both in free-living forms and in lichen symbioses. Dinitrogen fixation by aquatic species has been measured in subarctic (61) and antarctic lakes (316), and crusts of blue-green algae have been observed in such disparate locales as the Australian and North American deserts (131, 443, 482) and on ice-free areas of Antarctica, where "algal peat" deposits of up to 15 cm depth are found (236, 655). Desert algal crust organisms have been shown to fix $^{15}N_2$ (457, 458). Active $N_2$ fixation by blue-green algae was detected at temperatures down to 1° (94) and activity with other species has been observed at 54° in North American hot springs (657). In ecosystems of the tundra and Arctic

areas, $N_2$-fixing lichens and nodulated plants play a dominant role. Included in the latter are at least ten species of legumes (94), but the non-legume alder occurs with greatest frequency in many northern climates. The average annual increment of $N_2$ fixed by Alaskan alder stands was recently evaluated at 15.6 g/m$^2$ (132).

Non-legume symbionts are frequently dominant among dune (466) and other pioneer vegetations (149, 165, 697), as demonstrated in the case of *Ceanothus*, which typically is among the first vegetation to reappear following California forest fires. In the normal course of plant succession following glacier recession, various nodulated $N_2$-fixing species are prevalent; these include *Dryas*, *Hippophaë*, and *Shepherdia*, as well as such legumes as *Oxytropa* and *Hedysarum* (293). Alnus dominates in an early stage which lasts about 50 years, during which time accretion of nitrogen has been estimated at 6.2 g/m$^2$ × yr (466). The distribution of some of the non-legumes is extensive (68); *Podocarpus*, for example, grows abundantly on four continents in climates varying from jungles near sea level to mountain regions above 3000 meters (259). Free-living bacterial diazotrophs probably do not contribute greatly to $N_2$ fixation in marginal areas, though bacterial fixation has been indicated in the rhizosphere activity of *Ammophila*, a dune plant (296).

d) Marine

The extent of $N_2$ fixation in the seas and fresh waters is probably the least defined factor in global $N_2$ fixation. The blue-green algae are the primary agents of aquatic $N_2$ fixation, but localized contributions from bacteria, particularly photosynthetic species, occur. Inland lakes have been analyzed with increasing frequency as the $C_2H_2$ assay method has become more widespread (285, 594; Table 3-6). In temperate regions the most prevalent $N_2$-fixing fresh-water blue-green algal species are *Gloeotrichia*, *Aphanizomenon* and *Anabaena*, and in tropical regions *Anabaena* and *Anabaenopsis* are most common (658).

Marine $N_2$ fixation has not yet been extensively examined, but here too pertinent investigations are increasing. Incorporation of $^{15}N_2$ by samplings of *Trichodesmium* blooms was reported earlier (199) and $C_2H_2$ reduction by this species has recently been demonstrated (95); though they are short-lived, the blooms of this planktonic blue-green alga alone could account for significant amounts of marine $N_2$ fixation since they are known to cover millions of hectares of surface in such widely separated areas as the Sargasso Sea and Red Sea, which was in fact named for these blooms (452). DUGDALE (198) calculated that a single bloom could fix as much as 1000 metric tons of $N_2$ per day. *Nostoc* species occupy vast areas on Pacific coral reefs and are well represented among the phytoplankton of the Indian and Atlantic Oceans as well (658). Another blue-green alga associated with marine $N_2$ fixation is *Calothrix* which grows profusely on rocky shores and is estimated to fix about 2.5 g N/m$^2$ × yr (658).

Though the blue-green algae appears to dominate aquatic $N_2$ fixation, $N_2$-fixing bacteria have also been observed in these habitats. Recent Japanese studies have shown unidentified $N_2$-fixing bacteria to be widely, but unevenly distributed throughout the Pacific, being particularly numerous among *Trichodesmium* (453). BROOKS, PUTNAM and BREZONIK (82) attributed the $N_2$ fixation they observed in estuary water to bacteria rather than algae. Additionally, bacterial diazotrophs in association with marine angiosperms have been estimated to fix as much as 240 g N/m$^2$ × yr (547). In fresh-water habitats, photosynthetic bacterial diazotrophs are well known, and heterotrophic species have been identified in rice paddies (333) and lakes (393).

Of pertinence are recent studies (80, 249, 297, 298, 363, 381, 746) which emphasize the presence and distribution of denitrifying bacteria in fresh- and salt-water habitats. To the extent that this activity occurs in aqueous environments, a roughly corresponding magnitude of $N_2$-fixing activity can be assumed to maintain a balance. Without a substantial annual rate of marine denitrification, MARTIN (452) has estimated that marine nitrogen input processes would exceed outgo processes by about 70 × $10^6$ metric tons/yr.

*2. Comparison with Previous Estimates*

The value of 175 × $10^6$ metric tons for the annual global rate of biological $N_2$ fixation is higher than previous estimates in the literature. For example, HUTCHINSON (325) estimated the annual rate to be between 8 and 105 × $10^6$ metric tons, and this evaluation, particularly the upper limit, has been quoted frequently. The lower limit was based on the estimated amount of combined nitrogen entering the oceans from land drainage; it was considered that the land-to-sea flow of nitrogen represented a unique transfer in the nitrogen cycle, a premise which now appears to be unjustified. The upper limit was based on an estimate from lysimeter studies in the U.S.A. of 0.67 g $N_2$ fixed by asymbiotic bacteria/$m^2$ land area × yr; symbiotic $N_2$ fixation was considered to increase this, but the rate was modified downward by an equal amount to arrive at an estimate of 0.7 g $N_2$ fixed/$m^2$ × yr that would be representative for the entire world land area, and not just temperate North America. A similar value had been developed earlier by HUTCHINSON from a rather involved interpretation of the nitrogen: phosphorous ratio in lakes (324). The final figures include no component for marine $N_2$ fixation.

DELWICHE and STOUT (164) estimated in 1964 that biological $N_2$ fixation on land was 5 times greater than industrial $N_2$ fixation; since the latter rate was given as about 15 × $10^6$ metric tons/yr, the estimated annual rate for biological $N_2$ fixation was about 75 × $10^6$ metric tons. No basis was given for this evaluation, and no estimate for marine activity was made. More recently, DELWICHE estimated annual inputs of 14 × $10^6$ metric tons for legume crops, 30 × $10^6$ for other terrestrial agents and 10 × $10^6$ for marine diazotrophs for a global rate of 54 × $10^6$ metric tons/yr; the values are indicated to be correct within a factor of 10.

HAUCK (299) recently tabulated literature values for annual biological $N_2$ fixation rates according to major ecosystems as follows (range in g N/$m^2$ × yr): crop land, 0.7-2.8; non-legume pasture, 0.7-11.4; grass-legume pasture, 7.3-86.5; forest, 5.8-59.4; paddy, 1.3-9.9, and waters, 7-25. No attempt was made to evaluate a weighted average value for each ecosystem or to develop a global total. The rates given in Table 3-5 are relatively conservative in comparison.

From the foregoing it is clear that estimates of the global $N_2$ fixation rate have of necessity been little more than best guesses neither helped nor hindered by a strong data base. Gradually this base is forming, and if the present estimate has any virtue beyond those made earlier, it is because of the new insights which the developing literature on $N_2$ fixation rates has provided.

The topics covered in this section, including the nitrogen cycle and the magnitude of nitrogen fixation, were discussed recently with particular reference to aquatic aspects (284).

# Part II. Biochemistry and Biophysics

In slightly more than a decade our knowledge of $N_2$ fixation at the molecular level has progressed from almost nothing to the point where both the reaction and the proteins which catalyze it can be described in considerable detail. The reductive nature of the reaction is established beyond question, and reaction requirements, inhibitors and parameters are well delineated. The peculiar involvement of ATP and the unique group of substrates reduced by nitrogenase indicate that it is an unusual, complex enzyme having little obvious precedent in enzymology. The term nitrogenase still has more functional than structural significance, since catalytic activity has not yet been established for any structurally defined enzyme species. However, the Mo-Fe protein and the Fe protein - the components of nitrogenase - are individually well characterized. These contain metal, as their names indicate, and acid-labile sulfide as well. Separately, they are catalytically inert; in combination they catalyze all reactions attributed to nitrogenase.

Nitrogenases from one source after another have been shown to conform to a common pattern, dispelling early speculation that $N_2$ fixation may proceed by fundamentally different mechanisms among the wide variety of organisms that can fix $N_2$. Probably nothing emphasizes their similarity so much as the ability of Mo-Fe proteins and Fe proteins from various sources to form functional nitrogenases when recombined across taxonomic lines. The extent of compatibility is brought out in Table II-1, which summarizes available data. It should be pointed out that in most of the reported recombinations the Fe protein fraction was contaminated with at least a small amount of Mo-Fe proteins and a positive result represents an enhancement rather than the restoration of activity; additionally, the relative amounts of the two fractions used in a given cross were seldom optimized for activity.

Although the data in Table II-1 are not complete with respect to all possible combinations, it is clear that: 1. homologous crosses were almost always more reactive than heterologous crosses; 2. the greater the spread in aerobic character between the sources of the two components, the less likely that an effective nitrogenase was formed, and 3. a surprisingly high proportion - about two thirds - of heterologous crosses were active, attesting to the compatibility of the nitrogenase proteins. Almost all crosses between components from facultative anaerobic, photosynthetic and aerobic bacteria and symbionts gave low to substantial activity, while those involving one component from an anaerobic bacterium were almost always inactive. A particularly interesting coupling is the one between the proteins of the blue-green alga *Anabaena* and those of the photosynthetic bacterium *Chloropseudomonas*, though the cross-reactivity pattern is not complete (639). The other photosynthetic organism tested, *Rhodospirillum*, failed to provide an active Fe protein, but yielded an Mo-Fe protein which behaved like those from the facultative and aerobic organisms (57).

Table II-1. Cross-reactivity of nitrogenase proteins[a,b]

| Source of Mo-Fe protein | | Source of Fe protein: Cp | Kp | Bp | Ce | Av | Ac | Mf | An | Rj |
|---|---|---|---|---|---|---|---|---|---|---|
| Bacteria | | | | | | | | | | |
| Anaerobic | Cp[c] | + | - | + | | - | | | | |
| Facultative non-photosynthetic | Kp | - | + | | | + | | + | | |
| | Bp | - | + | + | | + | | + | | |
| Facultative photosynthetic | Ce | | | | + | | | | - | |
| | Rr | - | + | + | | | + | + | | |
| Aerobic | Av | - | + | + | | + | | | | + |
| | Ac | | | | | | + | + | | |
| | Mf | - | + | + | | | + | + | | |
| Blue-green Alga | An | | | | + | | | | + | |
| Legume | Rj | - | | + | | + | | | | + |

[a] Refs. 57, 169, 285, 369, 508, 639.
[b] + indicates positive cross-reactivity
[c] Cp *Clostridium pasteurianum*; Kp *Klebsiella pneumoniae*; Bp *Bacillus polymyxa*; Ce *Chloropseudomonas ethylicum*; Rr *Rhodospirillum rubrum*; Av *Azotobacter vinelandii*; Ac *Azotobacter chroococcum*; Mf *Mycobacterium flavum*; An *Anabaena cylindrica*; Rj *Rhizobium japonicum* bacteroids

To some extent the lessons learned in nitrogenase work with one diazotroph have been applicable to nitrogenases from other sources. In practice, however, this often meant providing only general guidelines, and experimental success depended primarily on how well the peculiarities of a given organism were understood and dealt with. In the biochemical and biophysical studies of nitrogenase, *Clostridium* and *Azotobacter* have been the charter organisms, and most of the available data on nitrogenase were obtained initially and most comprehensively with the enzymes from these two bacteria. The fact that one is a strict aerobe and the other a strict anaerobe enhances the value of the pooled data obtained with them, particularly with regard to the many similarities found. Increasingly, descriptive data are entering the literature from studies with other organisms. Most conspicuous is the recent genetic and analytical work with *Klebsiella*, but additionally, progress with the nitrogenase systems of blue-green algae, nodules and other bacteria is noted. In general the data from different sources serve to further emphasize the close similarity which exists among the nitrogenases of the various diazotrophs.

The chapters in this section will include a brief, generally chronological, account of the steps which have led to our present conceptualization of the nitrogenase reaction, discussions of the physical and chemical characteristics of nitrogenase and of its catalytic acitivity, and an evaluation of the mechanistic implications of these data. Additonally, the nature and role of proteins associated with nitrogenase will be discussed.

# An Equation for the Nitrogenase Reaction

Research in the biochemistry and biophysics of $N_2$ fixation has progressed along two separate but related lines. One deals mainly with the catalytic activity of the enzyme nitrogenase, while the other is concerned chiefly with its physical and chemical characteristics. Productive research in the former was initiated with the preparation of active cell-free extracts in 1960, but characterization work did not get well under way until into the mid-1960's. By then the over-all reaction characteristics had become reasonably clear, though the detailed reaction mechanism was not established, nor is it yet. Since the mid-1960's, understanding of the nitrogenase reaction has become increasingly refined, and major advances in defining the physical-chemical nature of the enzyme have been made possible with the availability of pure nitrogenase proteins for analysis. Until now it has been feasible and also fruitful to pursue these two areas rather independently; however, as experimentation becomes more definitive and bears more directly on the mechanism of nitrogenase action, these areas seem destined to overlap increasingly as they blend toward the ultimate goal, the description of nitrogenase catalysis in terms of molecular structure.

The present concept of the nitrogenase reaction was constructed over a period of at least ten years from numerous independent contributions of several laboratories. The initial cell-free work of CARNAHAN, MORTENSON, MOWER and CASTLE in 1960 (120), which unequivocally defined ammonia as the first free product of $N_2$ fixation, may be taken as a starting point, though actually the place of ammonia as the "key intermediate" had been established beyond reasonable doubt by earlier kinetic studies of $^{15}N_2$ incorporation by whole cells. These studies and other work with intact cells had produced a considerable literature by the late 1950's (108, 717). Most of the diazotrophs known today had been identified, and nutritional studies showed the importance of certain micronutrients for $N_2$ fixation, particularly Mo and Fe. The inhibitory nature of CO, $H_2$, NO and $N_2O$ had been discovered and studied, and a unique relationship between hydrogenase activity and $N_2$ fixation was established. However, it was increasingly apparent that the potential of the available whole-cell test systems for biochemical research was limited and in fact reaching the point of exhaustion.

In recognition of these limitations, continuing efforts to extract nitrogenase and demonstrate activity *in vitro* had been pursued since the 1930's, but the results obtained were negative or at best inconsistent. This barrier was finally penetrated in 1960 with the demonstration of reproducible $N_2$ fixation in *Clostridium pasteurianum* extracts by CARNAHAN et al. (120). The work was rapidly confirmed by SCHNEIDER, BRADBEER, SINGH, WANG, WILSON and BURRIS (597), who additionally reported successful applications of the *Clostridium* methods to *Rhodospirillum rubrum* and by other means observed $N_2$ reduction with blue-green algae. The *Rhodospirillum* preparations subsequently proved difficult to reproduce, and the algal extracts, which were not as well defined nor as active as the bacterial, were clearly less attractive for enzymic exploitation. The *Clostridium* system with its excellent repro-

ducibility and high activity was recognized as the preferred experimental system, and for four years *Clostridium* preparations remained essentially the only test system used in the research aimed at purification of nitrogenase and definition of its catalytic activities.

## I. The Clostridium-Pyruvate $N_2$-Fixing System

The initial reaction mixture of CARNAHAN et al., which has such a marked stimulatory effect on $N_2$ fixation research, consisted of a crude extract of *C. pasteurianum* cells incubated under $^{15}N_2$ in the presence of pyruvate. Crucial to successful $^{15}N_2$ fixation was the use of high levels of pyruvate in the incubation mixture (α-keto-butyrate also functioned, but not as well). Effective extracts were obtained by rupture of frozen cells in a press or by autolysis of cells which had been dried anaerobically at elevated temperature, a technique whose effectiveness was subsequently rationalized by the discovery of the cold lability of nitrogenase. The activity of the *Clostridium* preparations was high enough to permit titrimetric assay of ammonia (484), which brought to an end the dependency on $^{15}N_2$ and the mass spectrometer for routine analysis.

Contrary to hopes and expectations, the detailed process of $N_2$ reduction was not readily apparent from an examination of the *Clostridium*-pyruvate system. The major route of pyruvate metabolism, oxidation via the so-called phosphoroclastic reaction, was reasonably well defined for the saccharolytic clostridia (Fig. 4-1); however, the relevance

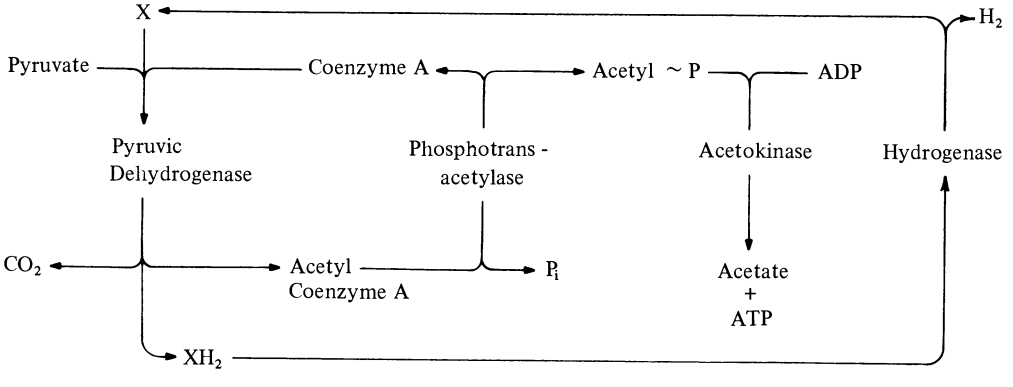


Fig. 4-1. Phosphoroclastic reaction of *Clostridium*, as it was known at the time that the first pyruvate-supported $N_2$ fixation was demonstrated with *Clostridium* extracts. The X was subsequently identified as ferredoxin. Acetyl ~ P is acetyl phosphate; $P_i$ is orthophosphate

of this reaction sequence, if any, to $N_2$ reduction was not at all clear. Acetyl phosphate was known to accumulate in the $N_2$-fixing reaction mixtures, but not in amounts equivalent to pyruvate disappearance, and it was suggested that the preparation contained enzymes capable of further metabolizing acetyl phosphate (120); acetokinase was known to be present in the extract (485), but the presence of functional levels of ADP was unsuspected, and the significance of

the acetokinase present was not readily apparent. The observation that about 100 moles of pyruvate were metabolized for each mole of $N_2$ reduced suggested in fact that the relationship between $N_2$ reduction and pyruvate might be in an obscure, minor pathway or that $N_2$ reduction was coupled to the phosphoroclastic reaction via a metabolic transient, possibly unknown.

Fractionation studies of $N_2$-fixing extracts of *Clostridium* and examination of the phosphoroclastic reaction led to the discovery of the electron carrier ferredoxin (p. 153) as the component marked X in Figure 4-1 (290, 494). The role of ferredoxin in $N_2$ fixation was demonstrated subsequently when MORTENSON (487) and D'EUSTACHIO and HARDY (170) showed that *Clostridium* preparations from which ferredoxin was removed fixed $N_2$ only when it was restored. Ferredoxin was thus established as a link between pyruvate metabolism and nitrogenase. Pyruvate metabolism could not be replaced, however, by alternate systems for reducing ferredoxin, such as an $N_2$→hydrogenase→ferredoxin system, and it was apparent that the support of $N_2$ fixation by pyruvate was more complex than (or something other than) simply the provision of reductant via ferredoxin.

## II. ATP

The complete explanation for the ability of pyruvate to support nitrogenase so effectively was finally achieved with the discovery that nitrogenase activity required ATP and that ATP was produced in the *Clostridium*-pyruvate system by the phosphorylation of ADP catalyzed by acetokinase at the expense of acetyl phosphate. The small amount of ATP (or ADP) that was fortuitously introduced into the reaction mixture in the bacterial extract was sufficient to support vigorous nitrogenase activity, since it was constantly recycled through the entire incubation. This is illustrated in Figure 4-2, which shows the dual role of pyruvate in providing both reducing power and energy for nitrogenase activity.

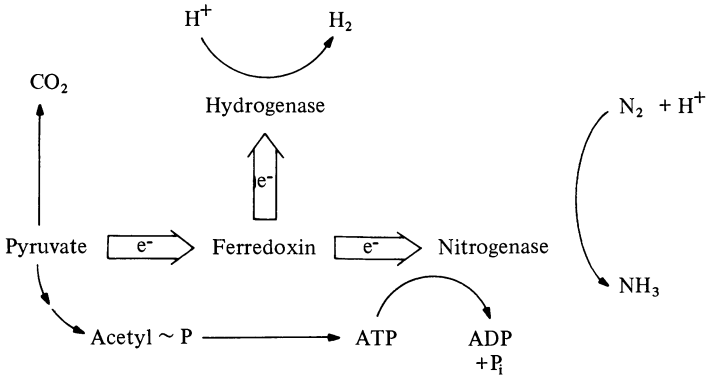


Fig. 4-2. The dual role of pyruvate in support of $N_2$ fixation as initially conceived. The oxidation of pyruvate provides energy, which reaches nitrogenase as ATP, and electrons, which reach nitrogenase via reduced ferredoxin; some electrons are drawn off in the $H_2$-forming action of *Clostridium* hydrogenase. The $H_2$-evolving activity of nitrogenase, initally unsuspected, is not shown

The discovery of the ATP requirement initiated a new productive surge in $N_2$ fixation research, which was then beginning to show signs of foundering as the promising early work appeared increasingly difficult to extend. McNARY and BURRIS had provided an early clue to the ATP requirement when they concluded from inhibition studies that "high energy" phosphate was required for $N_2$ fixation (462). Elsewhere the literature was confusing: inhibition of ATP had been demonstrated by CARNAHAN et al. (120); MORTENSON (485) described alternate explanations for the results of McNARY and BURRIS, and MORTENSON, VALENTINE and CARNAHAN (494) reported that glutathione could replace phosphate in $N_2$-fixing reaction mixtures, a replacement which precluded ATP formation. Against this background of uncertainty, reports by HARDY and D'EUSTACHIO (278) and MORTENSON (486) appeared which unequivocally identified ATP as a reactant in nitrogenase catalysis. In these mutually supportive investigations the successful replacement of pyruvate by the recombination of a ferredoxin-reducing system and an ATP-generating system was demonstrated. The use of regenerating systems, rather than substrate levels, was preferred for both reactants, since substrate amounts of reduced ferredoxin were experimentally impractical and high levels of ATP rapidly gave rise to an inhibitory ADP concentration. The regenerating systems were most easily provided by adding acetyl phosphate to the *Clostridium* extract and incubating in the presence of $H_2$. In such a reaction mixture reduced ferredoxin was constantly regenerated at the expense of $H_2$ via hydrogenase, and ATP was provided at the expense of acetyl phosphate via acetokinase and endogenous ADP; alternatively, ATP was furnished by supplementation with creatine phosphate and creatine kinase (Fig. 4-3).

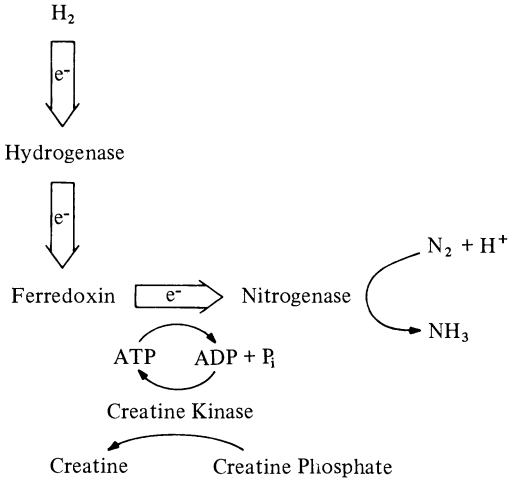


Fig. 4-3. *Clostridium* nitrogenase supported by $H_2$ via hydrogenase-ferredoxin and by an ATP-generating system

In retrospect it is clear that the early attempts to obtain $N_2$ fixation *in vitro* had been frustrated not so much from the inability to extract a functional enzyme as from ignorance of the substrate requirements of the extracted enzyme. Failure to appreciate the need for ATP particularly must have sent many functional nitrogenase preparations down the laboratory drain. Considering the basic simplicity of its make-up and the complexity of its action, the *Clostridium* - pyruvate

system emerges as a rather fortuitous mixture. In addition to nitrogenase, the *Clostridium* extracts contained sufficient pyruvic dehydrogenase, phosphotransacetylase, acetokinase, hydrogenase[4], ferredoxin, coenzyme A, $Mg^{2+}$, phosphate and ATP or ADP. The only additive required was pyruvate. A final attribute of this system is its ability to generate ATP at a constant, low level, a condition subsequently shown to be optimal for support of nitrogenase, since it circumvents the inhibitory effects of ADP accumulation and high ATP concentration. It is perhaps not surprising that other systems failed to meet all of the requirements for $N_2$ fixation *in vitro*; the wonder is that even one of them did.

## III. Azotobacter Nitrogenase and Dithionite

During the developmental work on the *Clostridium* system, continued attempts to obtain $N_2$ fixation with extracts of other organisms were made. As indicated above, initial successes with preparations from *Rhodospirillum* were difficult to reproduce and work on blue-green algae was not pursued. GRAU and WILSON (257) reported the successful application of the *Clostridium* techniques to *Bacillus polymyxa* in 1963, but the results could not be reproduced until 1970 (232) in a study which indicated that early attempts to confirm (726) had failed because the extracts lacked sufficient ADP or ATP.

A single report of marginal fixation by *Chromatium* extracts (23) was not followed up. Successful results were reported with *Azotobacter* preparations (518), but attempts to confirm these were unseccessful, and it is difficult to reconcile these early reports with the present understanding of *Azotobacter* nitrogenase.

It was not until after the *Clostridium*-pyruvate work was completed that significant progress was made with extracts of other diazotrophs, and the roots of the work that followed can be traced back to those initial investigations that defined the reaction requirements of nitrogenase. The first and most far-reaching advance to follow the *Clostridium* work was the extension of $N_2$ fixation to *Azotobacter* preparations by BULEN, BURNS and LeCOMTE (518).

The successful *Azotobacter* system consisted of a crude French press extract of $N_2$-grown *A. vinelandii* supplemented with phosphate, $MgCl_2$, a creatine kinase-creatine phosphate ATP-generating system and an extract of *Clostridium*, heated to destroy nitrogenase activity. The latter was included to supply possible cofactors for nitrogenase, but was subsequently found to be functioning as the electron donating system for the *Azotobacter* nitrogenase. When incubated under an atmosphere of $N_2$ and $H_2$, this mixture produced significant amounts of ammonia. Attempts to replace the *Clostridium* electron-generating system

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[4] Hydrogenase may appear unnecessary, even undesirable, but its presence is required in the system to regenerate sufficient oxidized ferredoxin for pyruvate oxidation to proceed: nitrogenase turnover requires about 4 or 5 moles of ATP per mole of electron pairs (in the form of reduced ferredoxin), and thus needs the ATP output of 4 or 5 moles of pyruvate to utilize the ferredoxin output of only one mole of pyruvate; without hydrogenase to serve as an alternate electron acceptor for ferredoxin, this imbalance would soon lock all ferredoxin in the reduced state and prevent further oxidation of pyruvate.

with a less cumbersome reductant led to the demonstration with preparations of both *Azotobacter* and *Rhodospirillum* that dithionite could support $N_2$ reduction fully as well as $H_2$ and the heated *Clostridium* preparation did (91). The reaction mixture used in these experiments - extract supplemented with dithionite and a creatine phosphate-creatine kinase ATP-generating system - has become a fairly standard reaction mixture for *in vitro* assays of nitrogenase; it appears to be universally effective for nitrogenase and has been used successfully with preparations from at least sixteen different sources (Fig. 4-4). Other early studies with *Azotobacter* preparations established a nitrogenase requirement for $Mg^{2+}$ (98), and this was subsequently shown to be satisfied, though less effectively, by $Mn^{2+}$, $Co^{2+}$, $Fe^{2+}$ or $Ni^{2+}$ (97).

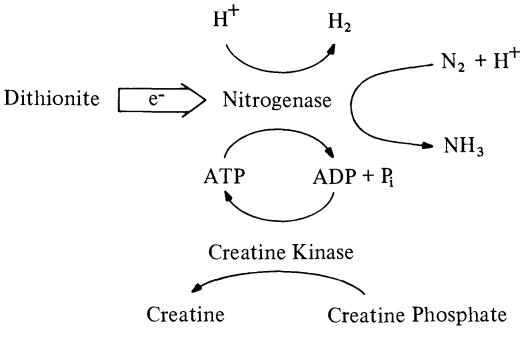


Fig. 4-4. Nitrogenase activity supported by dithionite and an ATP-generating system, showing the concomitant evolution of $H_2$

## IV. $H_2$Evolution and ATP Hydrolysis: Nitrogenase Activity without $N_2$

Application of dithionite to the *Azotobacter* nitrogenase preparation revealed that this system evolved copious amounts of $H_2$ in the absence of $N_2$, that ATP was required for this $H_2$ evolution, and that even in the presence of $N_2$, some $H_2$ was evolved, as shown in Figure 4-4 (91). Though the amount of $H_2$ formed constitutes a significant fraction of the reaction products, it does not generate a $pH_2$ sufficiently great to inhibit nitrogenase under normal assay conditions. Further analysis showed that CO, a potent inhibitor of $N_2$ reduction, had no effect on the ATP-dependent $H_2$ evolution activity; however, it was clear that this activity was intimately involved in nitrogenase enzymology and had little to do with the so-called "classic hydrogenase" of *Azotobacter* (p. 160; 89, 97, 98). When the use of dithionite was successfully extended to *Clostridium* preparations, the $H_2$-evolving reaction also was observed with the nitrogenase of that organism; this work with *Clostridium* further showed that ferredoxin was not essential for $N_2$ fixation by *Clostridium* nitrogenase. The ATP-dependent $H_2$-evolving activity was most clearly demonstrated with *Clostridium* preparations by incubating the dithionite-supported reactions under CO, which specifically inhibited the vigorous ATP-independent $H_2$ evolution catalyzed by the classical $H_2$-evolving hydrogenase present in the preparations; alternatively, interference from classical hydrogenase activity could be circumvented by removing ferredoxin from the preparations, since electron transfer from dithionite to hydro-

genase requires ferredoxin (96, 291). It became apparent that ATP-dependent $H_2$ evolution was catalyzed by nitrogenase, and therefore that nitrogenase possessed at least two active sites, distinguished by their responses to CO (93, 291).

The $H_2$-evolving activity was demonstrated with *Rhodospirillum* extracts (99) and has since been observed with nitrogenase preparations from all sources tested. In the early literature this function of nitrogenase was referred to variously as ATP-dependent $H_2$ evolution (98) and also as reductant-dependent ATPase (288), the latter terminology emphasizing the ATP utilization aspect of the reaction. Recognition of this function of nitrogenase explained the "$H_2$-requiring ATPase" of *Clostridium* preparations observed earlier by MORTENSON (486) and DILWORTH et al. (177): preparations incubated under $H_2$ hydrolyzed ATP (as determined by acetylphosphate disappearance) at a greater rate than those under argon; yet no $H_2$ was utilized according to manometric measurement. In retrospect it is now clear that this phenomenon was due to concommitant ATP hydrolysis and $H_2$ evolution by nitrogenase, the latter balanced by $H_2$ taken up and used in the sequence $H_2 \rightarrow$hydrogenase$\rightarrow$ferredoxin$\rightarrow$nitrogenase$\rightarrow H_2$.

The studies of ATP hydrolysis, $N_2$ reduction and $H_2$ evolution revealed a peculiar and unsuspected aspect of nitrogenase activity: $N_2$ was neither required for enzyme turnover nor did it even influence turnover rate. The enzyme appeared inert unless both ATP and a reductant were present, but given these, nitrogenase performed like a biological battery, providing a flux of electrons at the expense of the chemical energy of ATP. Catalysis appeared to be basically a matter of coupling ATP hydrolysis with electron transfer. With $H_3O^+$ ever present as an electron acceptor, yielding $H_2$, the flow of electrons was assured whether $N_2$ was present or not. In the presence of $N_2$ a portion of the electron flux was utilized for $N_2$ reduction, the balance, for $H_2$ evolution (Fig. 4-4). At saturating levels of $N_2$ approximately one electron went into $H_2$ evolution for every three that went to reduce $N_2$, so that in general, for each $N_2$ fixed, one $H_2$ was evolved. A relationship of primary interest was (and still is) the amount of ATP consumed per pair of electrons transferred. The value for this ratio varies among reporting laboratories (p. 114), but there is agreement that it is not influenced by $N_2$.

## V. Alternate Substrates for Nitrogenase

The formulation of the nitrogenase reaction reached its present state of maturity in the period 1966-1967 when it was expanded to reflect the ability of the enzyme to reduce substrates other than $N_2$ (and $H_3O^+$). Within a short period the reductions of the following substrates were reported: azide by SCHÖLLHORN and BURRIS (599) and HARDY and KNIGHT (289); $C_2H_2$ by DILWORTH (171) and SCHÖLLHORN and BURRIS (600); $N_2O$ and cyanide by HARDY and KNIGHT (289). Methyl isonitrile was added to the list by KELLY, POSTGATE and RICHARDS (372), and HARDY and JACKSON (286) showed the reduction of alkyne, nitrile and isonitrile analogs.

Like many other characteristics of nitrogenase the substrate versatility of the enzyme was not anticipated from earlier studies with intact organisms, though a low level of $N_2O$ utilization by soybean nodules and *Azotobacter* cells had been variously indicated and contraindicated (311, 504, 720); however, this early evidence was not sufficiently convincing to stimulate attempts to demonstrate $N_2O$ reduc-

tion with cell-free extracts when they became available. The discoveries of the various alternate reducible substrates developed instead from inhibition studies, since it was the ability of these compounds to inhibit $N_2$ reduction and $H_2$ evolution that initially brought them to notice.

In the case of $N_2O$, data obtained in the course of an early inhibition study (435) indicated some $N_2O$ reduction, but at a rate too low to elicit much attention or further work. Subsequently, however, after a detailed examination of the $N_2O$ inhibition pattern, it was concluded that $N_2O$ reduction might be a major reaction of nitrogenase, and further experimentation demonstrated this to be the case (287). The inhibition pattern had specifically shown that $N_2O$ did not affect ATP hydrolysis, but that it inhibited $H_2$ evolution. This appeared inconsistent with the concept of a constant ratio of ATP hydrolyzed per pair of electrons transferred, unless more electrons were being transferred than could be accounted for in the $H_2$ evolved. Accordingly, evidence for the reduction of $N_2O$ was sought and the production of $N_2$ (plus $H_2O$) from $N_2O$ was demonstrated (287). A detailed formulation of the nitrogenase reaction as it is currently conceived, showing the variety of reducible substrates and their products, is presented in Figure 4-5.

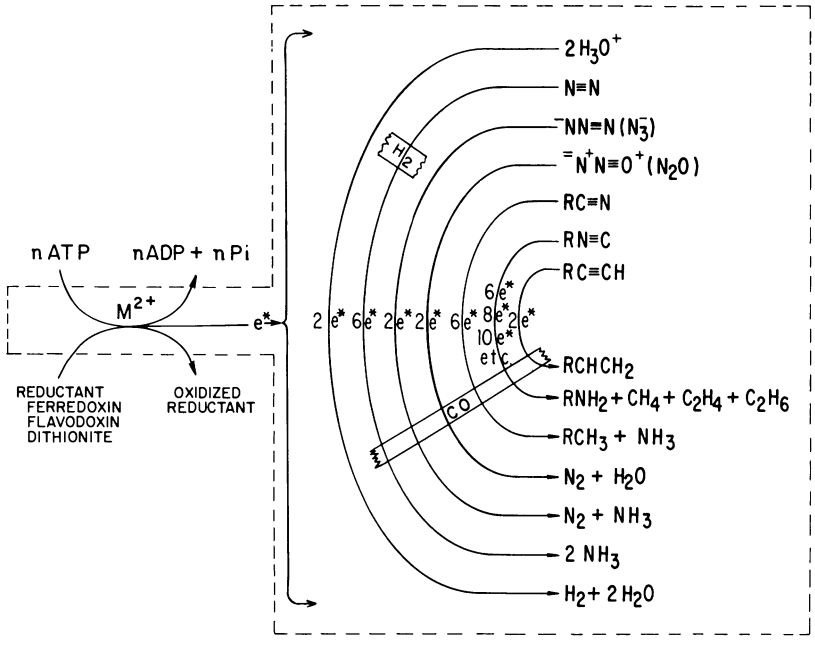


Fig. 4-5. The reactions of nitrogenase, based on utilization of ATP for formation of a strong reductant, or activated electron, e*, which can reduce $H_3O^+$, or $N_2$ and the other indicated species, subject to the $H_2$ and CO inhibition shown

## VI. $H_2$, $N_2$, Hydrogenase and Nitrogenase — A Special Relationship?

Throughout the course of research in $N_2$ fixation, $H_2$ has played a persistent role. Various involvements of $H_2$ and its metabolism have

been observed, nearly always unexpectedly, but often with significant impact on our conceptualization of the nitrogenase reaction. Some of these observations have already been referred to; others will be developed in the discussion that follows. It is difficult at this time to evaluate what common links, if any, unite these various observations involving $H_2$. They are discussed here topically to provide an added degree of perspective not readily apparent from the numerous isolated and specific references to the roles and effects of dihydrogen made in the following chapters.

The involvement of $H_2$ with $N_2$ fixation goes back almost 40 years to the early work of WILSON and colleagues, who for reasons of economy used $H_2$ as an "inert diluent gas" in kinetic studies with intact clover plants, only to discover that $H_2$ was not inert, but rather was a competitive inhibitor of $N_2$ fixation (see Ref. 717). The inhibitory nature of $H_2$ was confirmed with *Azotobacter*, and additionally it was demonstrated that this strict aerobe possessed a hydrogenase which was particularly evident when the cells were cultured on $N_2$. The growing suspicion of a relationship between hydrogenase and $N_2$ fixation prompted the screening of $H_2$-evolving organisms for an ability to fix $N_2$. These considerations plus the additional observation that $N_2$ inhibited the $H_2$ evolution of *Rhodospirillum* led KAMEN and GEST in 1949 to test this photosynthetic bacterium for $N_2$ fixation with the newly available $^{15}N_2$ (360). The positive results they obtained provided the first definitive evidence that $N_2$ fixation extended beyond *Azotobacter*, *Clostridium* and *Nostoc*, and also provided stimulation for further fruitful work in the testing and identification of diazotrophs.

It soon became apparent that all diazotrophs possessed hydrogenase activity, though this was not unique to them. An apparent inconsistency was resolved in 1957, when HOCH, LITTLE and BURRIS (310) demonstrated hydrogenase activity in soybean nodules by mass spectrometry after earlier efforts had given only negative results. In this work with soybean nodules both $H_2$ evolution and an exchange between $D_2$ and endogenous hydrogen donors, yielding HD, were observed. Further work (311) showed that $N_2$ was required for the exchange reaction to occur.

The involvement of $H_2$ carried into the work with nitrogenase *in vitro*, which became possible in 1960. Dihydrogen was shown to be an effective ultimate electron donor for nitrogenase via hydrogenase and ferredoxin, and the demonstration of ATP-dependent $H_2$ evolution by nitrogenase preparations indicated a direct $H_2$-generating function of nitrogenase. JACKSON, PARSHALL and HARDY (339) demonstrated that the $D_2$ exchange reaction was specific for the reduction of $N_2$, but not other substrates, and they simulated this activity with transition metal complexes of diazene and hydrazine. The specificity of $H_2$ inhibition for $N_2$ reduction has also been demonstrated.

The relationships between nitrogenase and dihydrogen, as they are currently conceived, have been enumerated (339) as shown below. The topics are described further in the pages indicated:

1. Evolution and/or utilization of $H_2$ by hydrogenase present in diazogrophs (p. 160);
2. ATP-dependent $H_2$ evolution (p. 121).
3. HD formation from $D_2$ and $H_2O$ (p. 126);
4. $H_2$ inhibition of $N_2$ reduction (p. 126).

# Nitrogenase: The Catalyst

## I. Procurement

### A. Factors Affecting Nitrogenase Biosynthesis

#### *1. Genetic Basis*

Until recently the molecular genetics of $N_2$ fixation was an almost completely unexplored area. Now, however, it has begun to yield to the classical techniques of bacterial genetics, and the foundation of this important aspect of $N_2$ fixation is taking form. The most noteworthy achievement to date, reported by DIXON and POSTGATE (180), is the transfer of genes for dinitrogen fixation *(nif)* from *Klebsiella pneumoniae* to *Escherichia coli*. Additionally, substantial progress has been made in the mapping of the *nif* genes (671, 673). Prior to this recent work on *Klebsiella*, genetic interest in $N_2$ fixation at the molecular level did not extend much beyond the procurement of nitrogenaseless mutants (below) or the development of improved *Rhizobia*-legume relationships (48). Such studies provided no insight into the nature of nitrogenase genetics *per se*. In contrast the investigations with *Klebsiella*, though just under way, have already yielded important results bearing directly on nitrogenase genetics and have established the base for continued investigation.

Of all the free-living diazotrophs which might be utilized for genetic studies, *Klebsiella* offered outstanding advantages. The genetic linkage map of *K. pneumoniae* is already partially constructed and known to be similar to that of the closely related and much studied *E. coli*. Aditionally, stable mutants of *K. pneumoniae* are readily obtainable, an important consideration for extensive genetic analysis. A further advantage is the facultative nature of *Klebsiella*, which permits much of the routine work with the organism to be performed under aerobic conditions.

The scheme used to produce $N_2$-fixing *E. coli* is illustrated in Figure 5-1. For the successful transfer of nitrogenase genes to *E. coli*, *K. pneumoniae* donor strains were selected which possessed two main characteristics in addition to $N_2$ fixation. These included a derepressed mutant I-like R factor and a high frequency transfer of the histidine *(his)* operon. Earlier studies (below) had shown that the R factor mediated conjugation and that the *his* marker was close to the *nif* genes of *Klebsiella*. A $his^-$, non-restricting and non-modifying strain of *E. coli* was chosen as the recipient organism. After mating, the donor strain was counterselected with streptomycin, and $his^+$ survivors were isolated on selective medium. These were shown to reduce $C_2H_2$ and to incorporate $^{15}N_2$ at rates commensurate with the rate of the donor *Klebsiella*. Standard bacteriological tests unequivocally established the organisms as *E. coli*. Though most of the $N_2$-fixing *E. coli* mutants proved to be unstable, the most active isolate was stable and retained its capacity to fix $N_2$ through several successive passages in ammonia-free

medium. A particularly interesting feature of the $N_2$-fixing *E. coli* is that nitrogenase biosynthesis by all isolates was repressed by ammonia, indicating that the regulatory genes as well as the structural, were transferred. This suggested that the transfers involved a *nif* operon or at least that the genes for these characteristics are closely associated.

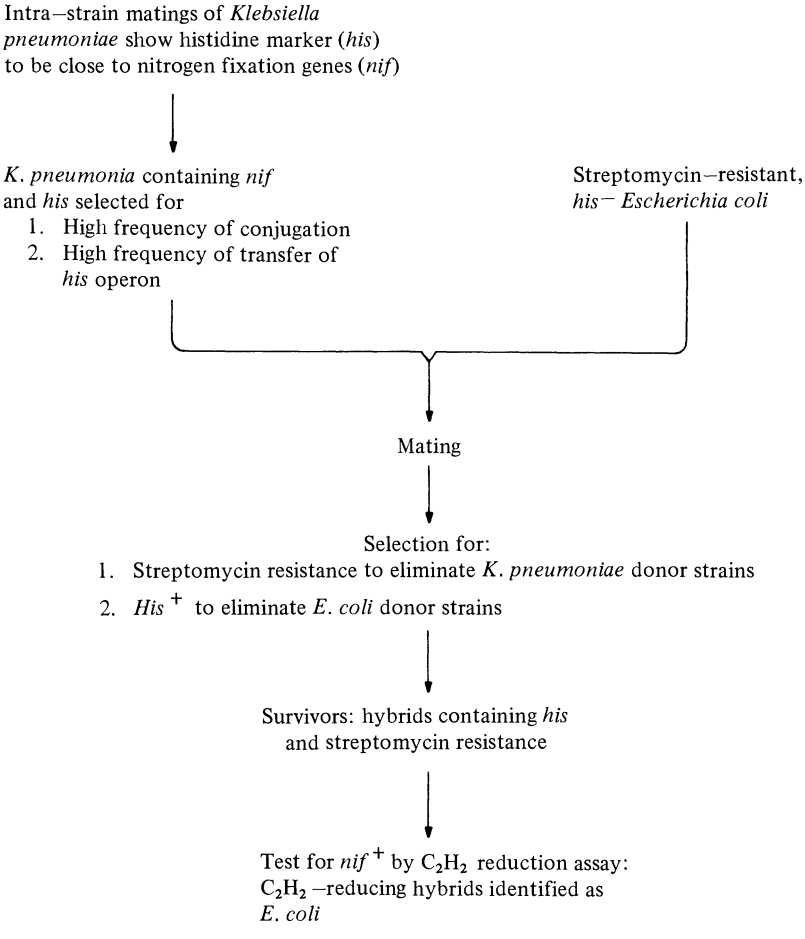
Intra–strain matings of *Klebsiella pneumoniae* show histidine marker (*his*) to be close to nitrogen fixation genes (*nif*)
*K. pneumonia* containing *nif* and *his* selected for
1. High frequency of conjugation
2. High frequency of transfer of *his* operon
Streptomycin–resistant, *his*– *Escherichia coli*
Mating
Selection for:
1. Streptomycin resistance to eliminate *K. pneumoniae* donor strains
2. *His* + to eliminate *E. coli* donor strains
Survivors: hybrids containing *his* and streptomycin resistance
Test for *nif* + by $C_2H_2$ reduction assay: $C_2H_2$ –reducing hybrids identified as *E. coli*

Fig. 5-1. Outline of procedure used by DIXON and POSTGATE (180) to transfer $N_2$-fixation genes from *Klebsiella pneumoniae* to *Escherichia coli*

Prior to the demonstration of *nif* transfer from *Klebsiella* to *E. coli*, intrageneric *nif* transfers had been demonstrated with *K. pneumoniae* by both transduction and conjugation. In both instances *nif*– mutants were shown to regain the ability to fix $N_2$ following receipt of genetic information from *nif*+ strains. The initial transductions were mediated by the generalized transducing bacteriophage P1 (672); subsequently, phage P2 and lambdoid phage 424 have been used (673). From their transductional analyses STREICHER, GURNEY and VALENTINE (672) concluded that the *nif* genes are located near the histidine operon

on the *Klebsiella* genetic linkage map, and the subsequent intrageneric conjugation studies (179) supported this conclusion. In the latter work the usefulness of the derepressed I-type R factor, R144drd3, for promoting conjugation was demonstrated. This factor was introduced into the donor *Klebsiella* strain from *E. coli*; *Klebsiella* mutants carrying the factor transferred genetic markers at high frequency, in contrast to the parent organism.

More than 150 *nif*⁻ *Klebsiella* mutants have been identified and are being used in transductional genetic linkage analyses designed to map the nitrogenase genes (671, 673). The high incidence of *nif*⁻ mutants suggests that a large number of genes may be involved in the establishment of $N_2$ fixation, but alternatively many of the lesions may relate only indirectly to nitrogenase. Nevertheless, most mutants failed to synthesize functional forms of either nitrogenase protein, as determined by *in vitro* recombination analyses for $C_2H_2$ reduction. Results obtained with mutants which did not possess a functional nitrogenase protein led to the tentative assignment of *nif*-95 as a gene for Fe protein and *nif*-23 as a gene for Mo-Fe protein (671). These transduced with the *his* marker at frequencies of about 60% and 40%, respectively (673).

Mutants unable to fix $N_2$ because of their failure to synthesize effective nitrogenase proteins have also been obtained from organisms other than *Klebsiella*. *Azotobacter vinelandii* mutants have been described in which effective Mo-Fe protein or Fe protein, or both of these, are lacking (231, 643, 644), and a single mutant of *Clostridium pasteurianum* has been shown to lack Mo-Fe protein activity (625). In the mutants which lacked only one protein, biosynthesis of the unaffected protein was repressed by ammonia. In the mutant lacking both proteins, the rate of reversion to wild type indicated that a single mutation had destroyed the ability to synthesize both proteins. Failure of a mutant to fix $N_2$ does not preclude the presence of nitrogenase in the organism, as shown with temperature-sensitive *Azotobacter* mutants which could not reduce $C_2H_2$ at 39° but yielded active cell-free extracts (37); the temperature-sensitive component was not identified, but a genetic lesion in a protein specifically involved in $N_2$ fixation was indicated.

## 2. *Culture Conditions*

### a) Metabolic Control

In all diazotrophs examined the development of nitrogenase is highly dependent on the nitrogen metabolism of the organism. This dependency has been most intensively studied in *Clostridium* (143, 167, 168), *Azotobacter* (670) and *Klebsiella* (539), where the results point to simple repression by ammonia as the most probable mechanism of control. As the product of $N_2$ reduction, ammonia is a metabolite universally (and preferentially) accepted by diazotrophs. Numerous other nitrogen metabolites are also capable of repressing nitrogenase (below), but whether these act directly or merely give rise to repressing levels of ammonia, as SORGER has suggested for repression by nitrate (642), remains unknown. The possibility that nitrogenase may be an inducible enzyme, specifically induced by $N_2$, has not received experimental support; however results remain equivocal because of the difficulty in maintaining cultures absolutely free of $N_2$ (167, 231, 447, 539, 625, 670).

In *Clostridium* (167, 231, 287, 289), *Azotobacter* (287, 288, 289, 641, 642, 670), and *Mycobacterium* (57), the lack of nitrogenase activity in repressed cells was shown by analyses of extracts to result from the absence of nitrogenase. Analyses of extracts showed that the two nitrogenase proteins of *Azotobacter* were degraded concomitantly as the cells became repressed following ammonia addition, while coordinate synthesis of the two proteins was demonstrated for cells undergoing derepression.

The unique electron paramagnetic resonance (e.p.r.) at a g-value of 3.7 [5] is characteristic of the Mo-Fe protein of nitrogenase (p. 87) and can be detected even in intact organisms. The signal has been used as a tracer to evaluate the appearance and disappearance of nitrogenase during derepression and repression (610). *Azotobacter* cells were examined in detail by this method, and it was also used with *Clostridium*, *Klebsiella* and *Bacillus*. In repressed *Azotobacter*, enzyme activity and the intensity of the e.p.r. spectrum both decayed more rapidly than did protein which cross-reacted with Mo-Fe protein antibody, suggesting that in repression the protein was first altered and subsequently destroyed. During derepression signal intensity and immunochemical reactivity developed in parallel, demonstrating *de novo* protein synthesis.

In earlier work with inhibitors of protein synthesis, STRANDBERG and WILSON (670) correlated the development of nitrogenase activity in derepressed nitrogenase with *de novo* protein synthesis, and a definite diauxic growth pattern has been observed with several organisms when cultured in the presence of $N_2$ with limiting quantities of ammonia (143, 447, 621, 670). Results with the algal diazotroph *Anabaena cylindrica* suggest that nitrogenase biosynthesis by this organism may be exceptionally sensitive to ammonia repression, even to the point where the ammonia formed in the course of $N_2$ fixation is sufficient to repress enzyme synthesis. Accordingly, high nitrogenase yields were encouraged by bubbling cultures for 40-48 hrs with a mixture of 95% Ar-5% $CO_2$ prior to harvest and extraction (635, 636). A similar regime was effective for *Plectonema boryanum* 594 (300), but the use of gases other than air destroyed, rather than stimulated, nitrogenase activity in cultures of *Gloeocapsa* (242).

The data covering repression by nitrogen metabolites other than ammonia present a confusing picture. The effectiveness of a given compound may relate to the ease with which it is assimilated (719), perhaps specifically to the ease with which it gives rise to ammonia accumulation; in the case of repression of *Azotobacter* nitrogenase by 2-methylamine, effectiveness depended on the source of carbon used in the culture, whereas repression by methylamine lacked this dependency (351). Nitrate repressed nitrogenase biosynthesis in *Azotobacter* (719) and *Anabaena* (71), though neither organism appears to be highly sensitive to this substrate; *Anabaena* obtained 50-65% of its cellular nitrogen from $N_2$ in the presence of 1.5 m*M* nitrate. In synchronized *Azotobacter* cultures continuous monitoring of nitrogenase activity by $C_2H_2$ reduction (404, 414) showed that additions of $NH_4^+$, but not other nitrogen sources, caused an immediate cessation of the increase in nitrogenase activity; in contrast, specific inhibitory effects by $NH_4^+$ are not normally observed *in vitro* (285; p. 114), and $NH_4^+$ addition to *Clostridium* growing on $N_2$ did not depress nitrogenase activity, but suppressed biosynthesis only (143).

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[5] Depending on the organism studied and the reporting laboratory, this resonance has been assigned a g-value of from 3.67 to 3.78.

Nitrogenase developed in glutamate-grown *Azotobacter* (719), *Klebsiella* (539), and *Rhodospirillum* (65), and in *Klebsiella* (539) and *Azotobacter* (643) grown on mixed amino acids, but not in *Klebsiella* grown on glutamine (539). Casein hydrolyzate has been reported variously to repress (719) and to have no effect (641) on the biosynthesis of *Azotobacter* nitrogenase, and a mutant showing diminished sensitivity to casein hydrolyzate was suggested to be partially constitutive for nitrogenase (641); if confirmed, this would be the first example of a constitutive nitrogenase.

Though combined nitrogen sources are generally considered to inhibit nitrogenase formation, levels too low to repress frequently stimulate its synthesis, as has been observed with *Mycobacterium* (58), *Clostridium* (285) and *Rhodospirillum* (507). It is not clear whether this stimulatory effect is directly on nitrogenase biosynthesis or on some other growth-related activity.

b) General Considerations

Because of the wide range of metabolic types found among the free-living diazotrophs, few general statements can be made concerning cultural conditions for the development of nitrogenase. It has long been apparant that certain diazotrophs are much more easily cultured on $N_2$ than others. Clearly, the major nutritional consideration for the development of nitrogenase is the exclusion of fixed nitrogen from the medium, though as indicated previously, total exclusion is not always necessary nor even desirable. Additionally, the mineral requirements are more exacting for growth on $N_2$ in that higher levels of Fe and Co are normally indicated and Mo is required, though it may be replaced in certain cases by V or W (110; p. 85). An increased sensitivity to $O_2$ must be allowed for, even among the aerobic organisms (p. 103). Certain of the diazotrophs, including the blue-green algae, the strictly aerobic and strictly anaerobic bacteria, grow on $N_2$ as on combined nitrogen with only the foregoing modifications. In contrast facultative non-photosynthetic diazotrophs fix $N_2$ vigorously only when cultured anaerobically (384), and the photosynthetic bacteria fix $N_2$ at a significant rate only when grown anaerobically in the the light (429).

Many of the free-living diazotrophs grow readily in liquid culture, and not surprisingly these have normally been chosen for $N_2$ fixation studies, particularly at the subcellular level. Continuous culture methods have been used successfully with numerous diazotrophs, including *Rhodospirillum* (507), *Azotobacter* (148, 414), *Mycobacterium* (58) and *Anabaena* (71), but more typically, diazotrophs are cultured in batches in volumes ranging from several to about 40 liters. Because of their requirement for light, the photosynthetic diazotrophs are usually restricted to the smaller volumes, but successful large-scale cultures have been obtained with others, including *Clostridium* (800 l) (271), *Mycobacterium* (120 l) (59) and *Azotobacter* (3000 l) (366). Harvested cells grown for extract preparation can normally be stored as frozen cell paste for prolonged periods prior to use (59, 107, 366, 369, 374, 386, 507, 721), though *Clostridium* is usually dried *in vacuo* at 30-50$^{o}$ (119, 120), for storage as dried cells.

Legume nodules are normally obtained from plants grown under controlled conditions in the greenhouse, in growth rooms or in field plots. Non-legume nodules, on the other hand, are usually obtained from natural sources, and work with disrupted nodules from these sources has not extended much beyond the suggestion of nitrogenase activity in crude homogenates, as with *Myrica cerifera* (621, 632).

## B. Extraction and Purification of Nitrogenase

A variety of cell breakage techniques are effective for the production of active $N_2$-fixing extracts (273), although sonication or disruption by shearing, as in the French cell, have had widest application. Dried cells, as used with *Clostridium*, readily undergo autolysis on wetting. Cell rupture of algae and of nodule bacteroids is frequently preceded by grinding or homogenization. Anaerobic conditions are necessary to preserve activity during cell rupture of all cells except *Azotobacter* (369), whose nitrogenase shows little $O_2$ sensitivity in crude extracts. A precaution found to be effective specifically in nodule extractions is the use of polyvinylpyrrolidinone, which appears to function by neutralizing interfering phenolic compounds (374, 394, 395).

In most work with nitrogenase *in vitro* the enzyme is at least partially purified prior to analysis. Since it is a complex of proteins rather than a single protein, the purification of nitrogenase can proceed along either of two paths: purification of the intact complex can be attempted, or alternatively the enzyme can be fractionated into its two dark brown protein components and each dealt with separately, then eventually recombined. In practice partial purification of the intact complex has been possible with several organisms, but preparations of greater than about 50% purity have been difficult to obtain. Best results have come from pursuing the individual proteins after fractionation, frequently preceded by a prior partial purification of the intact complex. The purest nitrogenase preparations, accordingly, are nitrogenases which are reconstituted from individually purified components; however, the absolute integrity of reconstituted nitrogenase *vs.* unfractionated nitrogenase is questionable, although it is qualitatively identical to unfractionated nitrogenase in all particulars examined (p. 105).

There is considerable range in the extent to which unfractionated nitrogenases from various sources have been purified. The most highly purified are those from *Azotobacter* and *Clostridium* , and these have been examined in greatest depth. Purifications of 6- to 10-fold are readily obtained from crude extracts which reduce sufficient $N_2$ for titrimetric assay of ammonia. This degree of purification yields preparations with specific activities 60-100 units $[N_2]$ [6] per mg protein which are estimated as 25-50% pure, attesting to the high level of nitrogenase (2-5% of soluble protein) initially present in the crude extracts. Recovery of activities of the *Azotobacter* and *Clostridium* enzymes through purification is normally about 50% or more. Purification of nitrogenase from other sources has proceeded more slowly, and analyses with these have often been performed with preparations which seldom exceeded a specific activity of 1 or 2 units $[N_2]$ per mg. Extracts from the photosynthetic bacteria appear to be particularly refractory to purification and have not yet yielded highly active preparations. Crude extracts of various diazotrophs have fre-

[6] At the Sanibel Island Colloquium on Biological $N_2$ Fixation, 1966 (reported in Science, Ref. 621) it was agreed to define a unit of nitrogenase as the amount of enzyme that can reduce 1 nmole $N_2$ to 2 nmoles $NH_3$ in one minute; specific activity was defined as units per mg protein. Because of the great variety of nitrogenase substrates activities are frequently reported in terms of these substrates or their reduction products, for example, as $C_2H_2$ reduced or $H_2$ formed. For clarification, the compound used for the activity measurement will be shown in brackets, as was proposed in Ref. 274. In general 1 unit $[N_2]$ is equivalent to 4 units $[H_2]$ or 3-4 units $[C_2H_2]$.

quently been used with no prior purification, and reports based on the performance of such preparations have appeared for *Azotobacter chroococcum* (366, 738), *Mycobacterium flavum* (58, 59), *Desulfovibrio* sp. (621), *Chloropseudomonas ethylicum* (218), *Anabaena cylindrica* (75, 300, 635, 636), *Plectonema boryanum* (300), *Gloeocapsa* sp. (242) and *Dolichos lab-lab* (Egyptian broad bean) (375).

The procedures employed for nitrogenase purification have been adapted from the standard methods of enzymology, and the enzyme has yielded at least in some degree to a wide range of treatments. More unique, perhaps, are the handling precautions which must be observed, since sensitivity to $O_2$ demands the application of anaerobic techniques, and the cold sensitivity of the Fe protein of most diazotrophs requires minimal exposure to low temperature. These sensitivities are most evident in highly purified preparations, but are sufficient even in most crude preparations to warrant the necessary precautions.

A particularly useful purification technique, which takes advantage of the acidic nature of nitrogenase, is the selective complexation and precipitation of the enzyme with a basic reagent followed by treatment with an acidic reagent to effect release. Protamine sulfate has been used most extensively as the percipitating agent, though polyethylene glycol and polypropylene glycol have replaced it in some recent work (334, 689); cellulose phosphate is the most common solubilizing agent. Cetyltrimethylammonium bromide has been used as a precipitant in one case (499), and RNA (499) and carboxymethyl cellulose (488) were also used to effect resolubilization. Purification by selective heat denaturation of non-nitrogenase proteins has been successfully applied to extracts of several diazotrophs. A summary of the purification procedures used with nitrogenases from various sources is given in Table 5-1.

Table 5-1. Methods used in purification of nitrogenase

| | Diazotroph | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Purification method | Av | Cp | Kp | Bp | Ch | Rr | Acy | Gm | Ll |
| Selective precipitation | | | | | | | | | |
| Protamine sulfate | + | + | | | | | | + | |
| Polypropylene glycol | + | + | | | | | | + | |
| Magnesium sulfate | + | | | | | | | | |
| Organic solvents | | + | | | + | | | | |
| Ammonium sulfate | + | | | | | | | | |
| Heat treatment | + | | + | + | | | | + | + |
| Gel filtration | + | | | | | + | | | + |
| DEAE cellulose chromatography | | + | + | + | | | + | | + |
| Calcium phosphate gel | | + | | | | | | | |
| Sucrose gradient centrifugation | + | | | | + | | | | |

References:
*Azotobacter vinelandii* (Av) 92, 102, 156, 264-266, 269, 327, 499, 523, 630, 677
*Clostridium pasteurianum* (Cp) 196, 285, 289, 463, 488, 492, 493, 689, 723
*Klebsiella pneumoniea* (Kp) 540
*Bacillus polymyxa* (Bp) 727
*Chromatium* (Ch) 721, 724
*Rhodospirillum rubrum* (Rr) 99, 507
*Anabaena cylindrica* (Acy) 301
*Glycine max* (soybean) bacteroids (Gm) 51, 52, 53, 216, 386, 690
*Lupinus luteus* (lupine) bacteroids (Ll) 374, 553

The nitrogenases of ten diazotrophs have been separated into their Mo-Fe protein and Fe protein fractions. These include *A. vinelandii* (92, 102, 107, 266, 368, 370, 499, 631, 678), *A. chroococcum* (368, 369), *M. flavum* (57), *K. pneumoniae* (56, 169, 200, 369), *B. polymyxa* (169, 369, 727), *C. pasteurianum* (145, 169, 285, 488, 492, 502, 689, 692), *C. ethylicum* (219), *A. cylindrica* (639), soybean bacteroids (46, 52, 53, 216, 386, 553) and lupine bacteroids (450, 553). Additionally, an apparent fractionation of *R. rubrum* nitrogenase was achieved, but the results remain equivocal, since only Mo-Fe protein activity was recovered (57). The most common method for separating the two nitrogenase proteins is DEAE column chromatography. Stepwise or gradient elution with NaCl or $MgCl_2$ solutions brings the Mo-Fe protein off the resin first. The elution of this protein is not sharp, and the subsequently eluted Fe protein fraction is frequently contaminated with the trailing edge of the Mo-Fe protein fraction. Quantities of more than 3 g of purified nitrogenase preparations have been readily fractionated in this way (102, 107). Though DEAE cellulose has had general application for nitrogenase fractionation, resins obtained from various sources have not always performed consistently (631). Gel filtration chromatography, particularly with the Sephadex gels, has also been successfully employed in fractionation.

There are two reports in the literature that support the fractionation of nitrogenase into three, rather than two, essential components. In one a zinc-containing protein was implicated in *Azotobacter* nitrogenase (357). In evaluating this claim it may be relevant that zinc was found to be non-specifically bound to both proteins of *Klebsiella* nitrogenase (371). The other report (681), which describes a third protein for *Clostridium* nitrogenase, has been specifically challenged (340). Both reports require verification.

Although the individual nitrogenase fractions lack catalytic activity, each possesses potential activity in the sense that it displays activity when recombined with its partner. It is frequently useful to evaluate this activity potential for a given fraction. This is normally done by saturating the fraction of interest with the alternate fraction and expressing the observed activity in terms of the weight

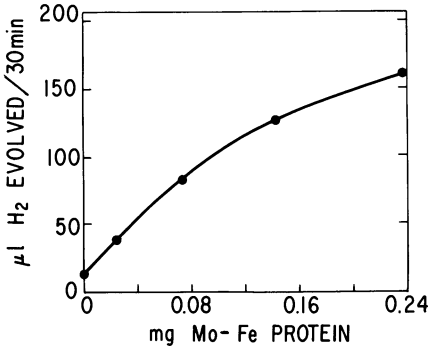


Fig. 5-2. Determination of Mo-Fe protein activity by titration against excess Fe protein. Each point represents a separate reaction. All reaction mixtures contained an excess of Fe protein, dithionite, an ATP-generating system, $MgCl_2$ and limiting amounts of Mo-Fe protein, as shown. No reducible substrates were added; $H_2$ production was measured manometrically

of only the limiting fraction. Because of variations in the potential activity of fractions, optimum values are determined most reliably from activity curves obtained by titrating each fraction against its partner, as shown in Figure 5-2 for Mo-Fe protein.

Purification of the individual fractions has yielded crystalline Mo-Fe protein from *Azotobacter* and homogeneous Mo-Fe and Fe proteins from *Clostridium* and *Klebsiella*. Partial purification of Fe protein from *Azotobacter* also has been reported, but little progress has been made with the individual nitrogenase protein from other sources. Specific activities in the range 1400-2600 units $[C_2H_2]$ per mg Mo-Fe protein and 1050-3100 units $[C_2H_2]$ per mg Fe protein have been reported for *Azotobacter* (102, 107, 499), *Clostridium* (145, 689, 692, 751) and *Klebsiella* (200) proteins. Though the Mo-Fe proteins of these bacteria appear to have been purified to homogeneity, the term must be used advisedly in view of the recent finding that *Clostridium* Mo-Fe protein preparations that were homogeneous by disc gel electrophoresis could in fact be separated into active and inactive proteins which differed in metal and $S^{2-}$ content (145, 751). To date *Azotobacter* Mo-Fe protein remains the only nitrogenase protein prepared in crystalline form (102, 107). Crystalline protein was obtained after first separating Mo-Fe protein from Fe protein by DEAE cellulose chromatography and then reducing the salt concentration of the Mo-Fe protein fraction. The simple expedient of diluting this fraction with water or weak buffer, preferably after bringing the pH to about 5.8 with acetic acid, resulted in the formation of crystalline protein.

## II. Properties of Mo–Fe Protein and Fe Protein

As pointed out in the previous section, nitrogenase has not yet been isolated in a pure state, though homogeneous or high purity preparations of the component proteins have been obtained from several sources. The physical and chemical characteristics attributed to nitrogenase itself are therefore largely based on deductions from studies of the individual Mo-Fe and Fe proteins. Of the numerous nitrogenases that have been fractionated, extensive purification and characterization have proceeded only with preparations from *Azotobacter*, *Clostridium* and *Klebsiella*. The results obtained with these organisms are for the most part mutually supportive, and the data obtained on a more limited scale with other sources are consistent with these. Continued work with the variety of other nitrogenases now obtainable should provide additional data for significant comparative analyses.

The characteristics of the nitrogenase proteins which have been examined so far are discussed below according to the source organism. These characteristics are then drawn together to provide composite descriptions of the two nitrogenase proteins. Some properties of the more completely defined proteins are compared in Table 5-2.

### A. Nitrogenase Proteins of Various Diazotrophs

#### *1. Azotobacter vinelandii*

a) Mo-Fe Protein

This protein possesses the unusual property of crystallizing out of solution when ionic strength is decreased (102, 107). The state change

Table 5-2. Comparison of properties of nitrogenase proteins from several sources

| Mo-Fe protein | *Clostridium pasteuranium* | *Azotobacter vinelandii* | *Klebsiella pneumoniae* |
|---|---|---|---|
| Molecular wt. ($\times 10^{-3}$) | 210-220 | 270-300 | 218 |
| Mo content | 2 | 2 | 1 |
| Fe | 11-12 | 30-38 | 17-18 |
| $S^{2-}$ | 11-12 | 26-28 | 16-18 |
| CySH | | 34 | 16-18 |
| Subunits: (no.) mol.wt. $\times 10^{-3}$ | (2) 50<br>(2) 60 | (4) 70 | (2) 51<br>(2) 60 |
| Est. purity (%) | 100 | 100 | 100 |
| Sp. act.: units $[N_2]$ per mg | 625-650[a] | 350-375[a] | 380 |
| Approximate turnover no.[7] | 70 | 50 | 83 |
| **Fe protein** | | | |
| Molecular wt. ($\times 10^{-3}$) | 55±5 | 20±6 | 66.8 |
| Fe content | 4 | - | 4 |
| $S^{2-}$ content | 4 | - | 4 |
| Tryptophan | 0 | - | 0 |
| Subunits: (no.) mol.wt. $\times 10^{-3}$ | (2) 27.5 | - | (2) 34.6±2.0 |
| Est. purity (%) | 100 | ? | 100 |
| Sp. act.: units $[N_2]$ per mg | 780[a] | 532[a] | 275 |
| Approximate turnover no.[7] | 21 | - | 9.5 |

[a] Evaluated from $C_2H_2$ reduction or $H_2$ evolution.

is the more striking because it occurs rapidly and because the crystals which form from the dark brown protein solution are white, or slightly yellow. By analogy with inorganic complexes the color change may reflect a change in ligand association in the chromophore environment (277). The crystals (Figs. 5-3, 4) are needle-like, and measure up to about 60μ long and 3μ at the widest point. They are extremely delicate, decomposing readily to a brown amorphous state on compression. The pure protein exists almost exclusively in amorphous or crystalline form at ionic strength of less than about 0.075 at 22° or 0.05 at 0°; the heat of solution was evaluated at -5.4 kcal per mole. As yet no other nitrogenase proteins have been crystallized.

A comparison of molecular weight values obtained by Mo analyses (107), sedimentation (107), and electron microscopy (264, 631, 648, 650) indicates that the protein exists under these analytical conditions as a dimolybdo species with molecular weight of 270,000-300,000 daltons. Iron, acid-labile sulfide ($S^{2-}$) and cysteine determinations were 30-36, 26-28, and 34, respectively, and the protein contained a normal complement of amino acids with an approximate twofold excess of acidic over basic residues (102). Specific activity was 1400-1500 units $[H_2]$, equivalent to about 350-375 units $[N_2]$, which corresponds to a turnover number of about 50 moles $N_2$ reduced per min per active center[7].

[7] Turnover number is defined as catalytic constant/No. of active centers; catalytic constant is moles product formed/min.×mole pure enzyme. For present purposes, active center of Mo-Fe protein is equated with 1 Mo and active center of Fe protein is equated with 2 Fe.

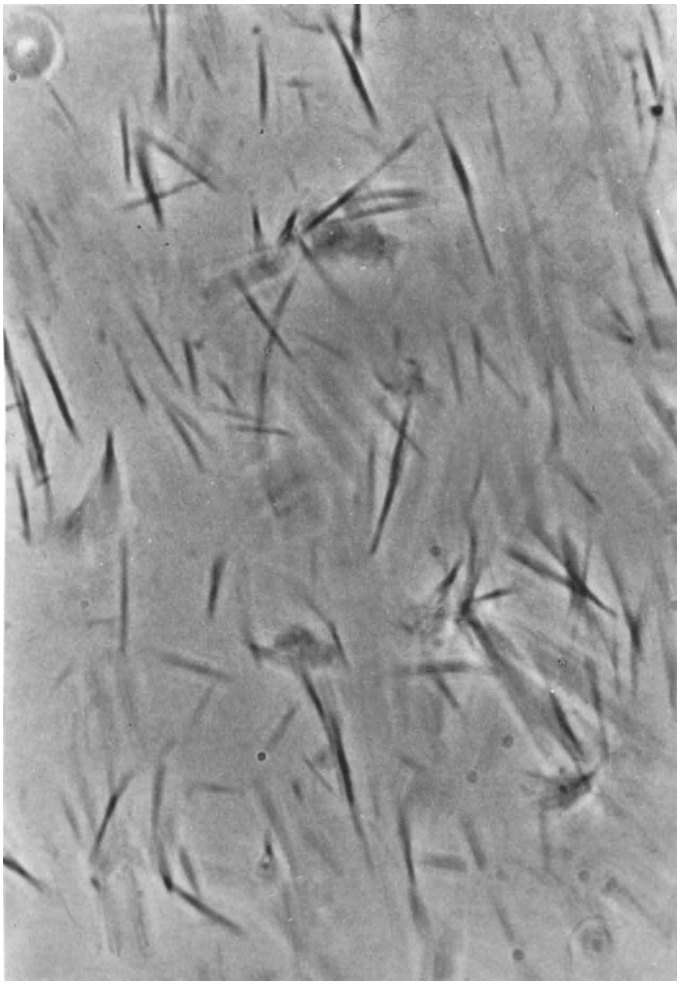

Fig. 5-3 a and b. Phase contrast micrographs of crystals of *Azotobacter* Mo-Fe protein. (a) Dispersed

The dimensions of the dimolybdo species were calculated to be 40 Å × 90 Å × 90 Å from electron micrographs like the one shown in Figure 5-4 and idealized in Figure 5-4g. These micrographs revealed a substructure of 4 similar non-spherical subunits calculated to have weights of about 70,000 daltons each. The centers of the subunits were electron dense, suggesting high metal concentration there. A molecular weight of 70,000 daltons was also indicated by polyacrylaminde gel electrophoresis of the sodium dodecyl sulfate-treated protein; only a single band was usually observed, even when the protein was also treated with iodoacetamide (650). Dissociation to smaller, different subunits may occur and is in fact indicated by DEAE chromatography of carboxymethylated Mo-Fe protein which partially separated two approximately equal components of similar, but not identical amino acid content (240).

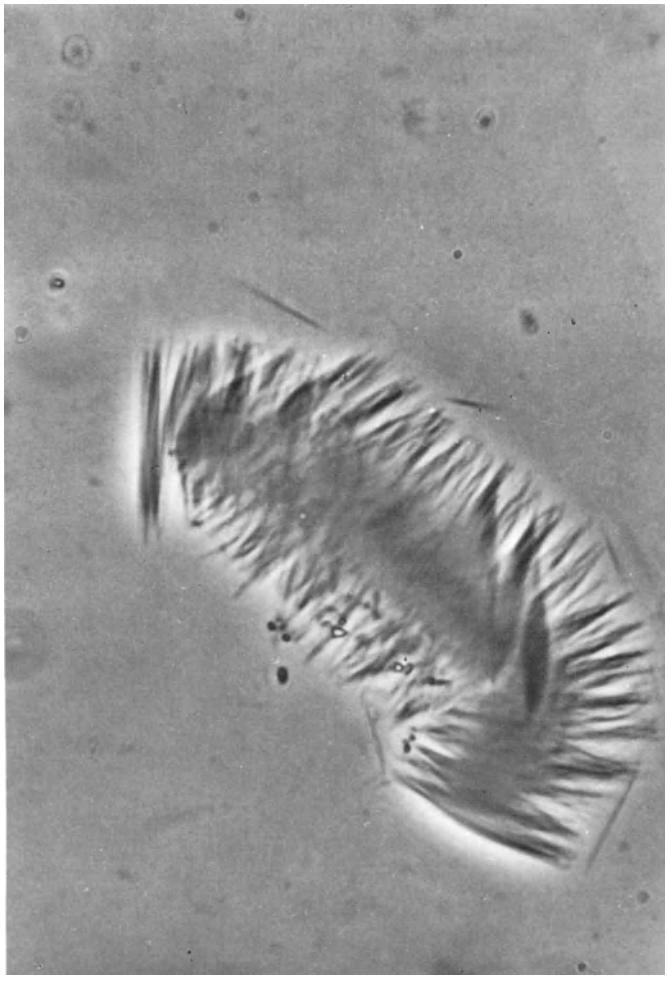

Fig. 5-3 (b). In transition between crystalline and amorphous states. Crystals are about 50μ long

In other electron microscopic analyses, low activity preparations were found to contain tetrads similar to those described previously, but the subunits were interpreted as spherical structures; on this basis a tetrad weight of 150,000 ± 25,000 daltons was estimated (631). Ultracentrifugal analyses of preparations heated at 60° for more than 10 min or subjected to 0.1-6 *M* urea or to elevated pH indicated dissociation of the protein to a maximum of six subunits. These treatments also dissociate Mo. A presumed subunit of the Mo-Fe protein, obtained by electrophoresis of a preparation of unknown activity, contained about three Fe per $S^{2-}$, but had no Mo (678).

There is substantial indirect evidence that *Azotobacter* grown on media supplemented with V instead of Mo produce a nitrogenase containing V in place of Mo. The activity of such preparations has been extensively analyzed (36, 100, 461), but there is no agreement on the role of

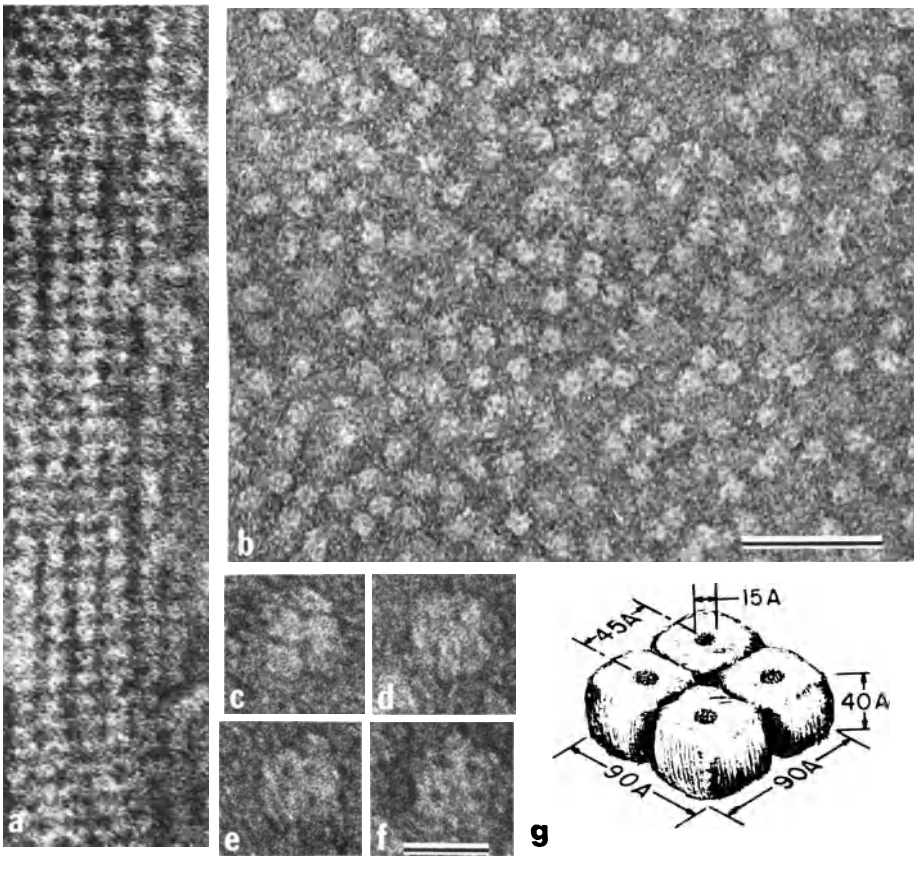


Fig. 5-4 a to g. Electron micrographs showing *Azotobacter* Mo-Fe protein in (a) crystalline form, (b) in 0.15 M ammonium formate, 70μg protein per ml, (c-f) enlargment from (b); (g) idealized version of (c-f)(Ref. 650). The bar in (f) is 100 Å

V. According to one interpretation V functions in the binding and reduction of reducible substrates (100); alternatively, it has been suggested that V simply stabilizes a Mo-deficient form of the protein (36). Attempts to isolate a V-Fe protein have been unsuccessful, but these have yielded a protein which appears to be an apo- or denatured form of the suspected protein. The isolated protein was precipitated by antiserum to Mo-Fe protein and was similar to Mo-Fe protein in solubility characteristics and amino acid analyses, but lacked biological activity, V, Mo, $S^{2-}$, and contained only about one half of the Fe found in the Mo-Fe protein (101). The u.v.-visible spectrum was similar to that of a Mo-Fe protein incubated for a protracted period in dithionite. Presumably, the difficulty in isolating a functional V-Fe protein lies in the instability of the molecule.

The u.v.-visible spectrum of crystallized Mo-Fe protein solubilized in 0.25 *M* NaCl is undistinguished except for a shoulder at about 412 nm. Molar absorbancy values are 470,000 at 280 nm and 85,000 at 412 nm based on a molecular weight of 270,000 daltons; the absorbancy per Fe at 280 nm was 14,700 based on 32 Fe. On reduction with dithionite the shoulder at 412 nm shifts to about 420 nm and becomes more

distinct, and slight absorbance appears at 525 and 557 nm (107). Absorption in the 410-420 nm region is also characteristic of modified Mo-Fe proteins, including the Mo-free protein and the presumed apoprotein from V-nitrogenase preparations already described; neither urea nor sodium-*p*-chloromercuribenzoate altered the spectrum of the former sample (678). Though the relevance of the absorbancy in this region is uncertain, it appears that the peak is most distinct and shifts furthest toward the red in samples of poor or questionable biological integrity. The spectrum of purified protein was unaffected by CO (677).

Numerous other spectral techniques have been used to examine the *Azotobacter* protein. In several of these studies, including some of the more sophisticated ones, preparations of low or undetermined purity and activity were used, and it is difficult to evaluate their significance (337, 523, 677); results obtained with crystallized, highly active material can be accepted with more confidence.

The Mössbauer spectrum of the native resolubilized crystalline protein in the range 20-200° K consists of a large doublet ($\delta$ = 0.31 mm/sec relative to Fe metal[8], $\Delta E$=0.84 mm/sec) with a small shoulder on the high field side which probably has a matching component obscured by the main dipole (706). On treatment with dithionite, absorption with $\delta$= 1.30 mm/sec and $\Delta E$=3.04 mm/sec develops at the expense of the original dipole. The spectra indicate that almost all of the iron in the native protein is high spin $Fe^{3+}$ with a small amount (the shoulder)of high spin $Fe^{2+}$, while about half of the iron in the reduced form is high spin $Fe^{2+}$ and the remainder high spin $Fe^{3+}$. These Mössbauer spectra are consistent with those obtained with a preparation indicated to be an active $N_2$-fixing *Azotobacter* protein complex of very low activity (possibly due to deficiency of Fe protein (523). In the latter study the magnitude of the quadrupole split in the native protein was considered indicative of strong asymmetry of the ligand surroundings, and the reduced spectrum was interpreted in terms of an increase in the 3d-orbital population, and therefore an increase in the shielding influence of the d-shell; thus the reduced state was characterized by a strong attraction of ligand electrons to the d shells of the Fe, leading to an unusual oxidation state of less than 2. Magnetic susceptibility determination by Faraday cage and nuclear magnetic resonance (n.m.r.) techniques indicated 3 Bohr magnetons per iron (560).

The e.p.r. spectrum of *Azotobacter* Mo-Fe protein shows resonance at g-values of 2.01 and 4.3, and a particularly interesting and unique resonance is observed at g=3.67; on reduction the intensities of these signals are enhanced. Additionally, a signal at a g-value of 1.94, typical of reduced iron-sulfur proteins, has been observed, but not consistently (102, 156). The isolation from apparently homogeneous *Clostridium* preparations of a metal-deficient Mo-Fe protein with resonance at g=1.94 (p. 90) suggests a similar origin for the *Azotobacter* 1.94 signal, though the two signals show different temperature dependency. On exposure to air, the signal at a g-value of 2.01 intensifies and the signal at a g-value of 3.67 diminishes and finally disappears (Fig. 5-5). Treatment with dithionite reverses these effects but does not restore full signal intensities. Spin-labeling analysis of purified Mo-Fe protein treated with a derivative of *p*-chloromercuribenzoate containing an iminoxylic radical showed a strong exchange

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[8] To facilitate comparisons, all isomer shift values are shown relative to a value of 0.0 for Fe metal.

interaction, suggesting close proximity of SH groups and thus of metal atoms (assuming cysteine-metal associations) (677).

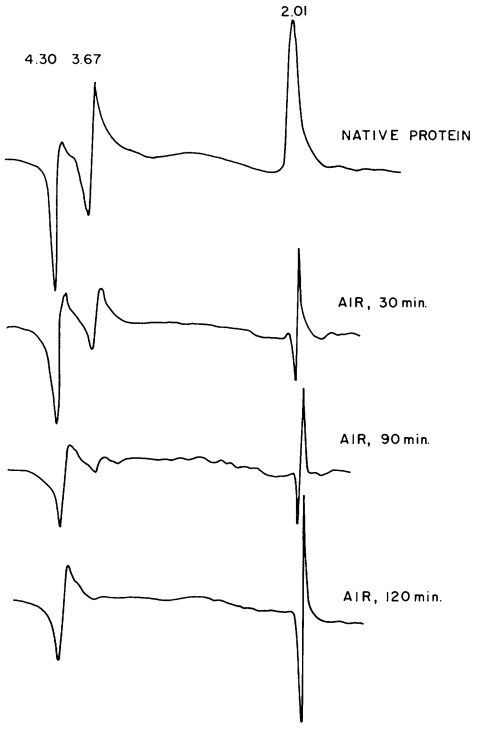



Fig. 5-5. Electron paramagnetic resonance spectrum of *Azotobacter* Mo-Fe protein and the effect of exposure to air; spectra were obtained at 4.2° K. The exposures to air were made at 22° C; g-values are indicated

An e.p.r. analysis of the Mo-free protein with a Fe:S ratio of 3:1 (678; see also Ref. 337 for e.p.r. studies on low activity preparations) detected no resonance in the spectrum of freshly prepared material, but resonance at g=1.94 and 2.01 was observed on reduction with dithionite; these resonances were attributed to Fe with a coordination number of 6 and tetragonal symmetry (678). An additional line at g=1.98 was observed upon acidification to pH 4 prior to reduction, or when the reduced sample was repeatedly frozen and thawed. Resonance at g=1.98 is often attributed to Mo (552), but Mo was not present in these samples. A slight increase in the signal at g=2.01 and decrease in the signal at g=1.94 were produced by adding $N_2$ plus ATP or by adding CO. The e.p.r. spectrum of the presumed apo-protein from V-nitrogenase preparations showed resonance only in the g=4.3 area, which was unaffected or slightly enhanced by dithionite (101).

Crystallized Mo-Fe protein is stable for at least a year when kept absolutely anaerobic and frozen at -15° or below in 0.25 *M* NaCl, 0.01 *M* Tris-HCl (pH 7.2). No loss of activity was observed after several days of storage at 0°. On exposure to air, activity was lost over a period of several hours at a rate dependent on protein concentration and agitation. Accompanying this loss was the disappearance

of the e.p.r. resonance at g=3.67, but negligible change in the visible spectrum was observed. Some restoration of activity in a partially inactivated preparation was provided by treatment with dithionite (105).

b) Fe Protein

Data on the chemical and physical characteristics of *Azotobacter* Fe protein are limited because of the unavailability of homogeneous preparations, and current information has been obtained with low activity and impure samples. A highly active preparation with specific activity of 2128 units $[C_2H_2]$ per mg was reported, but none of its characteristics was described (499).

Electron microscopy of purified preparations has been extensively used for evaluating the gross structure of the protein (264, 631, 648, 650). The dominant species observed is a particle identified variously as a sphere with diameter of 35-40Å (631) or as an elipsoid with dimensions 35 × 40Å (650). These occurred in aggregates of two, four and six particles, and in stacks of the six particle species, but were less frequently observed as isolated monomers (631, 650). Particles probably identical to these were observed as components of a more complex structure that was suggested to be an intact nitrogenase (264). Based on the observed shapes and dimensions of the apparent Fe protein monomer, its molecular weight was estimated at about 20,000 (631), 20,000±6,000 (264) and 24,000 daltons (650). An earlier value of about 37,000 daltons, based on a cylindrical shape, appears to be high (648), though this value compares with a molecular weight estimate of 30,000-40,000 daltons for a 2-Fe species based on the sedimentation rate observed with a preparation of about 70% purity (92).

Mössbauer analysis shows a single dipole with $\delta$=0.64 mm/sec and $\Delta$=0.84 mm/sec, indicating all Fe to be high spin $Fe^{3+}$; on treatment with dithionite this was completely replaced by a dipole with $\delta$=1.58 mm/sec and $\Delta E$=2.95 mm/sec, indicating complete conversion to high spin $Fe^{2+}$ (706). Close proximity of the Fe atoms was suggested from a spin-labeling experiment identical to that performed with Mo-Fe protein (p. 87; 677).

The protein is stable in the presence of $O_2$ in crude extracts (90), but becomes extremely $O_2$-sensitive when highly purified (92, 499). Similarly, in crude preparations the protein is unaffected by cold, but sensitivity to cold develops with purification (92, 370, 499); some protection was afforded by 10% ethanol (370). The inactivation by cold has been attributed to the loss of labile sulfide, which was reported to split off readily at $4^{o}$ C (679). The degree of sensitivity to $O_2$ and to cold is indicated by a loss of activity of more than 50% after being shaken in air for 2 min and a loss of about 75% after anaerobic storage for 20 hrs at 0-$1^{o}$ C (370). No loss of Fe occurred on anaerobic storage for 20 hrs in the pH range 5.5-9.5, presumably at room temperature (266).

*2. Clostridium pasteurianum*

a) Mo-Fe Protein

This protein has been purified to homogeneity as indicated by disc electrophoresis (145, 689, 751). The reported activities of 2500-2600 units $[C_2H_2]$ per mg, representing a turnover number for $N_2$ of about 70, are based on 2 Mo per molecular weight of 210,000-220,000 daltons (689, 751). In one case (751) protein shown to be homogeneous by electrophoresis was found to be heterogeneous with respect to active center(s), and substantial "impure protein" was removed by chromatography. As with the Mo-Fe proteins of nitrogenases from other sources, the role of the *Clostridium* Mo-Fe protein in catalysis remains unknown. It has been implicated in the binding of ATP (377, 491) and cyanide (87, 483, 490), but these claims have been disputed (56), and in particular ATP binding does not appear to occur (752).

Numerous molecular weight values have been reported, but a consistent value of 210,000-220,000 daltons has now been determined independently in two laboratories (689, 751). Metal content is indicated to be 2 Mo and 11-12 Fe and $S^{2-}$ per molecule; additionally, the presence of Mg (145, 377, 491) and Ca (145) has been noted. The "impure protein" mentioned above contains 2 Fe and 2 $S^{2-}$ per molecule of 220,000 daltons.

At concentrations below 5 mg/ml the Mo-Fe protein dissociates into particles of presumably 110,000 daltons, which separate into approximately equal amounts of 50,000 and 60,000 dalton components on passage through Sephadex G-100 (751). These subunits have also been obtained by treating the Mo-Fe protein with sodium dodecyl sulfate and then subjecting it to gel electrophoresis (511) or by gel filtration following treatment with mersalyl (701), and by treatment with $O_2$, dithiothreitol, EDTA, α,-α'-dipyridyl, urea, alkali and Chelex-100. The isolated subunits lack metal and $S^{2-}$, and they have not been successfully recombined to form an active protein. The preceding results show the dimolybdo species to be a tetrad composed of two 50,000 dalton and two 60,000 dalton subunits, a composition very similar to that observed for the *Azotobacter* Mo-Fe protein by electron microscopy (p. 84).

The e.p.r. spectrum of the protein isolated in the presence of dithionite showed resonances at g=2.01, 3.78 and 4.27 (145, 534, 751). Approximately 0.5 paramagnet per molecule was calculated, and $^{95}Mo$ studies indicated no Mo components in the spectrum. Addition of ferricyanide produced a series of spectral changes which were dependent on ferricyanide concentration, resulting finally in intense resonance at $g \perp = 2.00$ and $g \parallel = 2.09$. The resonance characteristics of the reduced protein were considered to reflect a polynuclear system of Fe atoms, exchange-coupled to yield a ground state of s=3/2; such a system would require at least 3 interacting Fe atoms, which could include both +2 and +3 valence states (534). It was suggested that the protein must be only partly reduced, since complete reduction or complete oxidation would not give rise to an e.p.r. spectrum (495). The "impure protein" gave resonance at g=1.94; its saturation characteristics were such that the resonance was clearly evident at 22° K, but was undetectable at 7° K.

The u.v.-visible spectrum of *Clostridium* Mo-Fe protein has no distinctive peaks or shoulders except for the 280 nm peak and perhaps a broad shoulder in the 300-400 nm region which varies with pH (342). A molar absorbancy of 302,000 at 280 nm can be calculated for the

210,000 dalton molecule from the data in Ref. 145. The absorbance per iron is about 25,000 based on 12 Fe; however, these values are uncorrected for "impure protein". The difference spectrum of reduced (native) protein minus air-exposed protein showed a minimum at about 360 nm and a maximum at about 435 nm, reflecting a color change from dark brown to reddish-brown.

Iron and sulfide were removed by acidification or by treatment with mersalyl followed by mercaptoethanol (490). Treatment with only mersalyl removed all Mo and $S^{2-}$, and an amount of Fe reported variously to be none (146) and 50-60% (483). Titrations with α,α'-dipyridyl indicated Fe to be in more than one valence state and/or environment (483). These titrations were also correlated with the ability of the treated protein to participate in catalysis when recombined with Fe protein (701), and indicated no activity loss on removal of 3 Fe atoms, linear loss of ATP-dependent $H_2$-evolving activity after the removal of more than 3 Fe atoms, linear loss of $C_2H_2$-reducing activity after the removal of more than 4 atoms of Fe, and diminution of ATP-hydrolyzing activity only after the removal of 7 Fe atoms.

The protein was inactivated by $O_2$ with 20-40% loss of activity after 5-10 min exposure to air; however, at least 60 min exposure was required for total inactivation (483). Spectral changes at about 360 nm and 435 nm, mentioned previously, occurred within seconds of exposure. Though the unpurified protein was reasonably stable to heat (195), the purified protein was destroyed by incubation at 60° for 10 min (340). The optimum pH for stability was between 6.8 and 7.4. It was relatively stable at 5° under $H_2$ for about a week and could be stored frozen for longer periods with losses of from 10-40% (492). Prolonged storage in liquid $N_2$ resulted in aggregation.

b) Fe Protein

Homogeneous Fe protein from *C. pasteurianum* has been prepared by two laboratories. In one case (689) activity was evaluated at 3100 units $[C_2H_2]$ per mg (equivalent to a turnover number for $N_2$ of about 21, based on the di-iron species); in the other case (341, 512) no activity measurement was reported. Activities of 460 units $[N_2]$ per mg and 2200-270 units $[C_2H_2]$ per mg were found previously for highly purified preparations (502, 534, 692). The homogeneous protein is brown to greenish in color (534) and contains a preponderance of acidic amino acids over basic. Its purity is based on the absence of tryptophan (341). Though early reports described a molecular weight of about 40,000 daltons per 2 Fe (341, 502), it is now apparent that a value of about 27,500 daltons per 2 Fe is more likely correct, as established by physical studies (512) and previously indicated by composition data which showed 2 μg atoms Fe per 28 mg protein (692).

The protein was isolated as a 55,000±5,000 dalton species which contained 4 Fe and 4 $S^{2-}$ and could be dissociated into two equivalent 27,5000±1,350 dalton subunits by treatment with sodium dodecylsulfate (512). The homogeneous preparations contained 16 SH groups per Fe, about half of which reacted immediately and the remainder more slowly with 5,5'-dithio-*bis*(2-nitrobenzoic acid) (341, 502). The protein was reported to bind Mg-ATP or ADP (87, 341); this was questioned on the basis of other analyses (56), but recent e.p.r. results support the earlier findings.

Early examination of the Fe protein by e.p.r. spectroscopy indicated that its only spectral characteristic was a signal at g=2 which oc-

curred on exposure of the protein to $O_2$ (341). It has now been shown that the reduced protein exhibits a rhombic 1.94-type signal with nominal g-values of 1.87, 1.94 and 2.06; slight resonance at a g-value of 4.3 is also seen. The spectrum was considered to suggest two different paramagnetic centers, though integration of the signal intensities indicated only 0.2 electrons per molecule of Fe protein (534). When the protein was incubated with $MgCl_2$ and ATP, the e.p.r. spectrum was altered, becoming nearly axial with g-values of 1.93 and 2.04, and the signal at g=4.3 increased in intensity. Titration with $MgCl_2$-ATP (1:1) produced spectral changes which were maximum after the addition of 2 Mg-ATP per 4-Fe protein species. Similar effects were observed when the protein was treated with $MgCl_2$ and ADP, GTP or β,γ-methylene ATP, but not α,β-methylene ATP. The altered spectrum could also be obtained by incubating the protein in 0.5-5 *M* urea, and this led to the speculation that Mg-ATP caused a change in Fe protein conformation (752). The possibility that Mg-ATP was binding to the Fe protein at an allosteric site was considered.

The protein is rapidly inactivated by $O_2$ and more slowly by exposure to temperatures around 0° (341, 501, 502). These effects are accompanied by alteration of the u.v.-visible spectrum; additionally, on oxidation an e.p.r. signal with a g-value of 2 develops. The protein is stable under an inert atmosphere for at least several days at room temperature or when stored in liquid $N_2$, where it is best kept in small (about 1 ml) volumes which can be frozen and thawed and warmed quickly to facilitate rapid passage through the critical inactivating temperature range. Exposure to air for 5 min resulted in complete inactivation with oxidation of half the $S^{2-}$, half the Fe ($Fe^{2+} \rightarrow Fe^{3+}$), and one fourth of the SH (341, 502). Loss of activity due to storage at low temperature amounted to 86% after 15 hrs at 0-1° (502). Accompanying this was an increase in the rate of reactivity with α,α'-dipyridyl, indicating an increased degree of exposure of reduced iron in the cold preparations. Some protection against cold inactivation was afforded by 10% acetone or alcohol (502) and by glycerol and dithiothreitol (340). The protein is stable to heating at 60° for 10 min in crude preparations, but not in purified form (340).

The effects of various treatments have been monitored by observing changes in the u.v.-visible spectrum, which for the native protein shows a narrow shoulder at 310 nm and a broad shoulder from 325 to 375 nm (502), but the significance of these changes is not clear. Preparations of native protein, which are yellow-brown, were bleached by mersalyl, causing a decrease in absorption at 375 nm as well as in the 260-280 nm region. Dithionite caused a slight increase in absorption through the entire visible range and was indicated to react with Fe protein with a stoichiometry of one dithionite per Fe protein monomer. After 5 min exposure to air, absorption decreased at 375 nm, increased at 312 nm, and a new peak appeared at 410 nm; subsequent incubation under $H_2$ in the presence of hydrogenase and ferredoxin partially reversed the spectral changes, suggesting electron transfer to Fe protein via $H_2 \rightarrow$hydrogenase$\rightarrow$ferredoxin$\rightarrow$Fe protein; however, activity was not restored. Exposure to cold produced decreased absorption at 310 nm and in the 400-600 nm region, while heating the preparation at 60° for 10 min caused an increase in absorption above 400 nm and a decrease below (340).

*3. Klebsiella pneumoniae*

In contrast to the literature of the *Azotobacter* and *Clostridium* nitrogenase proteins, which accumulated gradually through the years, the

corresponding *Klebsiella* literature consists essentially of a single extensive report (200). Application of the lessons learned with the two former organisms has resulted in comprehensive characterizations of the two *Klebsiella* proteins, which can now be compared with the *Clostridium* and *Azotobacter* proteins; the report also includes the only circular dichroism (c.d.) data for nitrogenase proteins.

a) Mo-Fe Protein

The molecular weight and subunit structure of the *Klebsiella* Mo-Fe protein are similar to those of the *Clostridium* Mo-Fe protein, but its Mo content and activity are only about half those found for the *Clostridium* protein. A mean molecular weight of 218,000 daltons was calculated from a variety of measurements, including disc electrophoresis, gel filtration, ultracentrifugation, amino acid composition and subunit composition. Metal content was determined after treatment with a metal chelating resin to remove spuriously-bound metal. One g atom Mo, 17-18 Fe, 2 Mg, 1 Cu, 1 Ca and 1 Zn were found per 218,000 daltons; Cd, Co and Mn were not detected. The acid-labile sulfide content was 16-18 g atoms per 218,000 daltons, and a equivalent number of cysteine residues was found. The amino acid content showed a preponderance of acidic residues over basic. When subjected to anaerobic isoelectric focussing, the protein yielded two bands with isoelectric points of 5 and 5.5. Activities of 1500 units $[H_2]$, 380 units $[N_2]$ and 1200 units $[C_2H_2]$ per mg were determined and represent turnover numbers of 327, 83, 262, respectively (200). Impure preparations bound ATP and cyanide, but no unique or specific binding was indicated, since control proteins showed similar binding (56).

Treatment of the Mo-Fe protein with β-mercaptoethanol and sodium dodecylsulfate produced two different subunits. These were separated by gel electrophoresis and their molecular weights were estimated at 51,300±1,700 and 59,600±1,900 by comparison of their mobility characteristics with standard proteins. The two subunits appeared to be present in equivalent amounts.

The u.v. spectra of the oxidized (ferricyanide or thionine-treated) and the reduced (dithionite-treated) proteins both have a major absorption peak at 277.5 nm and shoulders at 258.5, 269, 282 and 289 nm. In the visible range both proteins absorbed into the near infrared region, but the spectra have little character other than near 430 nm where the oxidized protein exhibits a shoulder. No differences were observed in the c.d. spectra of the oxidized and reduced proteins, both showing a single negative trough at 220 nm and possibly a second below 210 nm. The α-helix content was estimated at 38%.

The e.p.r. spectrum of the reduced protein exhibited g-values of 4.3, 3.7 and 2.01; additionally, weak resonance at a g-value of 1.94 was observed. Inactivation of the Mo-Fe protein by $O_2$ eliminated the resonance at g=3.7. Careful re-oxidation of reduced protein produced a complex series of reversible changes in which new resonance in the region of g=1.6-2.0 emerged as the resonance at g=3.7 disappeared.

The Mössbauer spectrum of the reduced homogeneous protein consists of at least three doublets characterized by $\delta$=0.90 mm/sec and $\Delta E$=3.05 mm/sec at 4.2° K, $\delta$=0.85 mm/sec and $\Delta E$=0.8 mm/sec at 4.2° K, and $\delta$=0.60 mm/sec and $\Delta E$=0.7 mm/sec at 77° K; broadening of the latter doublet occurs at 4.2° K, indicating half-integral spin. After inactivation by $O_2$, a doublet with $\delta$=0.60 mm/sec and $\Delta E$=0.9 mm/sec was apparent, and this behavior was interpreted in terms of spin-coupling

between the Fe atoms. These Mössbauer data (200) represent an extensive refinement over earlier Mössbauer results (371) obtained before highly purified protein became available.

The protein is irreversibly inactivated by $O_2$, losing 60% of its activity after 10 min exposure to 0.2 atm $O_2$ in Ar at 30°. Under anaerobic conditions, however, the protein was quite stable with no loss for up to 50 hrs at room temperature, pH 7.8. At 0° the protein is unaffected by pH in the range 4.5 to 9.2.

b) Fe Protein

The specific activity of homogeneous protein was 1050 units $[H_2]$, 275 units $[N_2]$ and 980 units $[C_2H_2]$ per mg. These values are equivalent to turnover numbers of 35, 9.5 and 33, respectively, substantially lower than those reported for purified *Clostridium* and partially purified *Azotobacter* protein.

Molecular weight evaluations by ultracentrifugation, gel filtration and amino acid composition led to a mean value of 66,800 daltons. Treatment with sodium dodecylsulfate caused complete dissociation into a single subunit species of 34,600±2,000 daltons. The behavior is analogous to that of the *Clostridium* Fe protein. Four atoms of Fe and acid labile sulfide were found per 66,800 daltons. Amino acid analysis indicated 10.6 cysteine residues per 66,800 daltons, no tryptophan, and an excess of acidic residues over basic. Isoelectric focussing of the reduced protein produced two major bands with pI values of about 4 and a minor band with a pI of about 5.9.

The u.v.-visible spectrum of the reduced (dithionite-treated) protein is undistinguished with only a single peak at 268 nm and a shoulder at 258 nm. Protein inactivated by $O_2$ exhibited a similar spectrum, but additionally had a shoulder at about 460 nm. The c.d. spectrum of the reduced protein showed a single negative trough at 220 nm and suggested an additional trough below 210. The spectrum of $O_2$-inactivated protein was similar, but indicated an $\alpha$-helix content of only 23%, compared with 33% for the reduced protein.

A weak e.p.r. signal at a g-value of 1.94 was detected with the dithionite-treated protein, and it was estimated that less than a single electron per 4-Fe species was involved. On exposure to $O_2$ all resonance disappeared, in contrast to the response observed with *Clostridium* protein. The Mössbauer spectrum of the pure Fe protein (200) showed distinct differences from those obtained earlier (371) with relatively impure material. The spectrum of the reduced pure protein consisted of a doublet at 77° K with $\delta$=0.70 mm/sec and $\Delta E$=1.1 mm/sec. At 4.2° K this broadened into a multiplet, indicating unpaired spin.

The *Klebsiella* Fe protein is particularly resistant to cold inactivation, with no loss in activity detected after 15 days of anaerobic storage at this temperature. Likewise, storage under $N_2$ at room temperature and pH 7.4 produced no ill effects. Like other nitrogenase Fe proteins the *Klebsiella* protein was extremely sensitive to $O_2$, losing over 70% activity when exposed to 0.20 atm $O_2$ in Ar at 30° for one min.

*4. Azotobacter chroococcum*

The nitrogenase activity of preparations of this organism has been examined extensively, but little information exists on the characteristics of the protein components. A Mo:Fe ratio of 1:12 was indicated for the Mo-Fe protein. The Fe protein was highly sensitive to $O_2$, while the Mo-Fe protein was relatively resistant; neither protein lost activity during storage in liquid $N_2$. On thawing, the Fe protein was kept at $0^{o}$ for 8 hrs without loss, perhaps indicating greater than usual cold stability; however, no specific activity values for the preparations used in these analyses were provided in the report (368). Characteristics of activity by the recombined fractions led to the suggestion that the sites for $H_2$ evolution and substrate reduction are on the Fe protein and that ATP plus electrons provided by the dithionite reduction of Mo-Fe protein react with the Fe protein to create a reduced site on the Fe protein (368). This suggestion seems inconsistent with data in the same paper which showed that the $C_2H_6$: $C_2H_4$ product ratio from $CH_3NC$ reduction was governed solely by the Mo-Fe protein in interspecies recombinations.

*5. Mycobacterium flavum*

Analyses of fractions with low specific activity indicated a ratio of 15 Fe per Mo in the Mo-Fe protein and an Fe content of 0.24% (w/w) for the Fe protein, from which a maximum molecular weight of 93,000 daltons per 4 Fe can be calculated (57). The Fe protein was more stable at $12^{o}$ than at $18^{o}$ and least stable at $0^{o}$; this protein also exhibited high sensitivity to $O_2$ in contrast to the Mo-Fe protein, which lost activity only after exposure to 0.1 atm $O_2$ for 60 min.

*6. Chloropseudomonas ethylicum*

Characterization work to date consists of an e.p.r. analysis of a Mo-Fe protein of unreported purity and activity (219). The spectra were recorded at $13^{o}$ K. In the reduced (dithionite) state resonance was observed at g-values of 1.94 and 2.00; treatment with ferricyanide or $O_2$ gave an oxidized spectrum in which the resonance at g=1.94 was replaced by resonance at g=2.04. Neither ATP nor cyanide affected the reduced spectrum when added separately, but when added together and in excess relative to dithionite, the oxidized spectrum was formed. In a similar experiment using Fe protein in place of Mo-Fe protein, no such spectral effects were observed. A reduced e.p.r. spectrum of Fe protein has been observed, but details have not been published (637). The results were interpreted to indicate that the Mo-Fe protein is the site of substrate activation and reduction.

*7. Soybean Bacteroids*

The proteins from this nitrogenase source have not yet been extensively characterized, but molecular weight, metal content and activity values have been reported. Homogeneous Mo-Fe protein recently has been prepared (334), and additional characterization can be expected. A molecular weight of 132,000 daltons was determined for this protein, and 0.6 atoms Mo, 15 Fe, and 14 $S^{2-}$ per molecule were found; activity was evaluated at 1000-1100 units $[C_2H_2]$ per mg. Amino acid analysis showed a preponderance of acidic residues over basic. In other work minimal molecular weights of 180,000 and 51,000 daltons were determined for a Mo-Fe protein containing one Mo and an Fe protein con-

taining one Fe, respectively (52). Activities of the Fe protein were 71 units $[C_2H_2]$ per mg and 115 units $[N_2]$ per mg (52, 386). Reactivation of nitrogenase and of Fe protein following loss of activity during storage or from $O_2$ exposure was accomplished by incubating the protein with $Fe^{2+}$, dithiothreitol and dithionite (52, 53). It has been suggested that the Fe protein functions as an allosteric effector, interacting with receptor sites on the Mo-Fe protein (53).

## B. Summary of Characteristics of the Nitrogenase Proteins

From the foregoing descriptions it is apparent that each diazotroph produces a nitrogenase with certain unique characteristics, but also with many properties similar to those exhibited by other diazotrophs. It is reasonable to assume that the unique qualities relate primarily to the specific manner in which nitrogenase is accommodated by the particular physiology and metabolism of each organism, while the characteristics possessed in common are more directly related to the $N_2$-fixing function of the enzyme. In this section these similarities are assimilated into generalized composite descriptions of each of the two nitrogenase proteins. These composites will not relate specifically to the proteins of any single diazotrophs, but they should have general application to those of all diazotrophs, and are intended to describe the major protein properties that are associated (though in ways still unknown) with the enzymic catalysis of $N_2$ fixation. In considering the characteristics of the nitrogenase proteins it is interesting to note that the Mo-Fe protein and Fe protein themselves share a number of similar properties in that both are acidic iron-sulfur proteins with no heme groups indicated, and both are dark-colored in solution and sensitive to $O_2$.

### *1. Mo-Fe Protein*

This component of nitrogenase is characterized primarily by its metal complement of Mo and Fe, which occur in a ratio of 1 to 12-16. Cysteine and acid-labile sulfide are present in amounts about equivalent to metal content. Molecular weight varies with the source organism, ranging from less than 150,000 to more than 200,000 daltons per Mo, and both mono- and dimolybdo species can occur. Electron micrographs show four discrete protein bodies per two Mo, and dissociating reagents produce four similar, but not identical subunits per Mo. In addition to Mo and Fe, one or two atoms of Mg or Ca may be present, and Mo may be replaced by V. It appears probable that Mo and Fe are involved in binding and reduction of substrates.

The u.v.-visible spectrum is undistinguished, as are spectra for other iron-sulfur proteins with more than 2 iron atoms, and in the absence of a strong reductant may exhibit no peaks except for the maximum at about 280 nm, where the high specific absorbance of 1.4-1.8 $A_{280}$ (1 mg protein/ml) reflects the presumed high u.v. absorbancy of the Fe-S prosthetic group; the absorption coefficient on a per Fe basis is 15,000-25,000. Iron, rather than Mo, is probably responsible for the absorption in the visible range as suggested by analogy with xanthine oxidase (77) and by the spectral similarity of normal Mo-Fe protein and the presumed apo-Mo-Fe protein isolated from extracts of *Azotobacter* grown on V. The spectrum is unaffected by CO. Increased absorption in the range 410-420 nm may result from reduction and/or denaturation.

The e.p.r. spectrum of the native or partly reduced protein shows resonances at g-values of about 2.0, 3.7 and 4.3. The most unique

feature of the spectrum appears to be the resonance at g=3.7 for which no counterpart has been described in the biochemical literature. The disappearance of this signal on exposure to $O_2$ correlates with activity loss, and it would seem to be the spectral component most uniquely associated with the catalytic role of the Mo-Fe protein. Additionally, oxidation enhances resonance in the g=2 area. The resonance at g=4.3 is enhanced on reduction with dithionite in contrast to the g=4.3 signal of rubredoxin, which diminishes on reduction.

A preponderance of high spin $Fe^{3+}$ in the native protein with about 50% reduction to high spin $Fe^{2+}$ or to an even lower valency on treatment with dithionite is favored by e.p.r. and Mössbauer data; a ground state of s=3/2 is suggested for the reduced protein, arising from exchange-coupled high-spin Fe in a rhombic site. The appearance of new e.p.r. signals in samples exposed to $O_2$ suggests significant interaction of metals in the native and reduced states and thus close spatial proximity; spin labeling experiments lead to the same conclusion. Little can be said at this point about the oxidation state of Mo. The absence of resonance assignable to Mo suggests that Mo is in the +4 or +6 state; alternatively, a +3 or +5 state can be considered in conjunction with strong metal-metal interactions which suppress normal resonances.

A full complement of the usual amino acids is found with a preponderance of acidic residues over basic. The high incidence of acidic residues is reflected in an isoelectric point in the pH 5 region, and clearly the acidic nature of the protein is the dominant property which has been utilized in purification procedures. Under normal storage conditions the Mo-Fe protein is reasonably stable, provided it is kept anaerobic; $O_2$ exposure leads to irreversible inactivation with a half-time for decay of 15-60 min.

*2. Fe Protein*

This smaller and less well-defined component contains equivalent Fe and acid labile sulfur, presumably in Fe-S groups which may be localized in two different environments. Depending on the source, this protein is purified as a 4-Fe species with molecular weight around 60,000 daltons or as a 2-Fe species with molecular weight of about 30,000 daltons. The 4-Fe species can be dissociated into two equivalent subunits of equal molecular weight. Electron micrographs reveal a basic structural unit which is round or ellipsoid and which is almost always seen in pairs or multiples of pairs. Its molecular weight is calculated at 24,000 daltons, and presumably it is a 2-Fe species. The protein lacks tryptophan, and like the Mo-Fe protein, it contains an excess of acidic residues over basic. The cysteine content is in excess of the Fe content. Two moles of ATP and Mg may bind to the 4-Fe species, and functionality as an effector in catalysis has been suggested.

In the presence of dithionite a weak e.p.r. signal of the rhombic g=1.94 type is observed; the integrated signal intensity indicates only about 0.2 moles electron per mole Fe protein (4-Fe species). Spectral changes reflecting a transition from rhombic to axial character occurs when $MgCl_2$ and ATP (or ADP or GTP) are added. The altered spectrum reaches maximum intensity after about two moles of Mg-ATP are added per 4-Fe species. On exposure of the isolated protein to $O_2$ a single spectral line at about g=2 develops. Mössbauer analyses show high spin $Fe^{3+}$ in the native state with conversion to high spin $Fe^{2+}$ on reduction; in conjunction with the e.p.r. observations, a strong

Fe-Fe interaction, as in other iron-sulfur proteins, is indicated in both native and reduced state, but not on oxidation by $O_2$. The u.v.-visible spectrum lacks distinguishing features other than a shoulder at 310 nm and broad absorption in the 325-375 nm region. Up to one third of the absorption at 280 nm and almost all of that above 350 are attributed to the Fe-S prosthetic group.

Among the more striking characteristics of the Fe protein are its extreme sensitivity to $O_2$ and its tendency to cold inactivation, particularly in the purified form. Low temperature and $O_2$ may cause changes in the visible spectrum; these differ with the treatment given and their significance is not clear. The appearance of e.p.r. resonance at g=2 on exposure to air suggests $O_2$ inactivation may involve a free radical; possible insight into the nature of cold inactivation is the observation that $S^{2-}$ is lost from the chilled protein. According to a recent theory for cold inactivation, this sensitivity could relate to a metabolic control mechanism (550).

## III. Properties of Nitrogenase

### A. The Question of Nitrogenase Composition

To early biological chemists examining $N_2$-fixing organisms, it was axiomatic that $N_2$ fixation was catalyzed by an enzyme. The term "nitrogenase" readily entered the literature, and its continued use in the functional sense remains fully justified even today. However, as the $N_2$ fixation literature developed, the term was applied increasingly in a structural or compositional context, acquiring the connotation more as a matter of semantic convenience than through design, but in any case with questionable justification. This casual extension of the term undoubtedly contributed to the concept, implicit in much of the literature, that there exists a structurally definable catalytic unit for $N_2$ reduction and, more precisely, that this unit is composed of Mo-Fe protein and Fe protein combined in a particular stoichiometric ratio:

$$x\ \text{Mo-Fe protein} + y\ \text{Fe protein} \leftrightarrow \text{Nitrogenase} \begin{pmatrix} \nearrow \text{Substrates} \\ \searrow \text{Products} \end{pmatrix}$$

The concept was strengthened by early cell-free work which showed that the two nitrogenase proteins stayed together through several purification steps; furthermore, when they were finally separated, neither protein possessed catalytic activity by itself. However, there is little direct support for this concept, and in fact some data seriously question its authenticity, as will be discussed below. Unfortunately, few investigations have dealt with the problem of nitrogenase composition in depth. This is partly because unfractionated nitrogenase preparations have not yet been purified sufficiently for meaningful structural studies. These preparations have been rather refractory to extended purification efforts, and most investigators have tended to purify only to the extent needed for confidence in reaction assays or to facilitate subsequent fractionation. Since pure Mo-Fe and Fe proteins are available, reconstituted nitrogenase would certainly appear to overcome the purity problem, but certain anomalies in the catalytic behavior of reconstituted preparations suggest that these may not be equivalent to the unfractionated enzyme (p. 105). An additional unknown factor is the amount and effect of pure but inactivated material which most Mo-Fe protein and Fe protein preparation may con-

tain. For example, it has been reported that low activity Fe protein or Fe protein partially inactivated by $O_2$ can inhibit the action of fully active protein (56, 751). Until these questions surrounding the reconstituted preparations are resolved, their utility for studies of a nitrogenase structure remains questionable.

Very likely electron microscopy will prove to be the definitive method for composition and structure, as has been the case for other enzymes. In the only reported study of its type, the gross composition of *Azotobacter* nitrogenase has been estimated from ultracentrifugal and electron micrographic analyses of purified, unfractionated enzyme (264). Sedimentation rates indicated a molecular weight of 340±60 thousand for a species indicated to contain 2 Mo and 30 Fe and assumed to be the $N_2$-fixing complex, though the possibility that it was the Mo-Fe protein alone cannot be excluded. Electron micrographs showed a complex consisting of a high molecular weight component of 8 subunits of 35,000±6,000 daltons and a low molecular weight component of 4 subunits, estimated at 20,000±6,000 daltons each. The high molecular weight component was suggested to be one dimolybdo Mo-Fe protein and the low molecular weight component was suggested to consist of two di-iron Fe proteins.

Titration experiments (p. 81), in which activity is plotted against the concentration of one nitrogenase protein while the concentration of the other is held constant, have on occasion produced sigmoidal curves when Fe protein was the varied protein. This observation, taken in conjunction with the inhibitory effect of excess Mo-Fe protein, has been interpreted to favor the existence of an active complex containing more than one Fe protein per Mo-Fe protein. Other activity data have been invoked in support of particular complexation stoichiometries on the assumption that the Mo-Fe:Fe-protein ratio that produces optimal activity defines the combining ratio of the proteins. The reasoning appears to be patently speculative, however, since the ratio for optimal activity does not necessarily have any significance for structure. It recently has been shown, for example, that *Clostridium* Mo-Fe protein activity is optimal when the molar ratio of Mo-Fe protein:Fe protein is 1:6, but that optimal Fe protein activity is obtained at a molar ratio of 1:2 (751). Nevertheless, activity studies have been interpreted to favor a molar ratio of 1:2 (343, 689, 692). In other activity studies, the optimum ratio varied according to the particular activity measured. Thus, for *A. chroococcum* preparations the optimum w/w ratio for $C_2H_2$ reduction for $H_2$ evolution was 2:1, but for isocyanide or cyanide reduction was 6:1 (368). Similar variation was observed with the *Mycobacterium* proteins (57). Based on differences in the optimum amounts of *Clostridium* Mo-Fe protein and Fe protein which were recombined for ATP hydrolysis activity and for substrate reduction activity, it was concluded that an ATP-hydrolyzing unit consisted of one Fe protein and at least two Mo-Fe proteins, but that a substrate-reducing unit consisted of at least two Fe proteins and an undefined number of Mo-Fe proteins (88). To the extent that these data have any significance for structure of a catalytic species, they clearly favor functional units of variable composition.

However, the most serious challenge to the concept of a structurally defined "catalytic nitrogenase unit" is provided by spectral data. Mössbauer spectra of the combined proteins appear to be only the sum of the spectra of the individual proteins (200, 706), and e.p.r. spectra are only slightly non-additive (495); in neither case is the creation of a new species indicated. Both of these spectral techniques also provide evidence that the individual proteins are not inert, as much of the nitrogenase literature implies. Both Mössbauer and e.p.r.

spectra show that the Mo-Fe and Fe proteins respond to reductant, and e.p.r. data show binding of ATP-Mg to *Clostridium* Fe protein (751) and to *Chloropseudomonas* Mo-Fe protein (219). The significance of these observations is not yet apparent.

General acceptance of the "catalytic nitrogenase unit" concept has resulted in little consideration for alternatives, though the early literature contains at least one exception (488). Many alternatives are in fact permitted within the limits of existing data. Simplified examples of reaction sequences which do not require a catalytic nitrogenase complex in the traditional sense are formulated below, where A and B represent the two nitrogenase proteins and x, y and z represent reductant, ATP and electron acceptor, not necessarily in that order. They are presented only to emphasize that numerous reaction sequence possibilities can be considered for nitrogenase reactivity. They have no support experimentally, but they are within the limits of existing data. Much more cannot be said for the traditional concept.

$$A + x \rightarrow Ax$$
$$Ax + B \rightarrow Bx + A$$
$$Bx + y + z \twoheadrightarrow B + \text{products}$$

$$A + x \rightarrow Ax$$
$$Ax + y \rightarrow Axy$$
$$Axy + B \rightarrow AxyB$$
$$AxyB + z \rightarrow A + B + \text{products}$$

$$A + B \rightarrow AB$$
$$AB + y \rightarrow Ay + B$$
$$Ay + x + z \twoheadrightarrow A + \text{products}$$

Clearly many sequences are possible, and it is anticipated that studies such as the kinetic, spectral and electron microscopic investigations that are now beginning to enter the literature will soon provide definitive answers to this most interesting problem of nitrogenase composition.

## B. Physical-Chemical Characteristics of Nitrogenase

In the previous section it was pointed out that unfractionated or recombined nitrogenase preparations have received little more than superficial analysis for structural determination. This holds true also for other physical-chemical studies generally, and for the same reasons of low purity and questionable protein integrity. In the limited investigations which have been undertaken, no unique feature of nitrogenase, other than catalytic activity, has emerged which could not be predicted by simply adding the characteristics of the Mo-Fe and Fe proteins. The characterization work reported for unfractionated nitrogenase consists almost exclusively of spectral analyses.

In some of the earliest investigations of crude *Clostridium* nitrogenase-pyruvic dehydrogenase preparations, $N_2$-induced changes were reported in the u.v.-visisble spectra (120, 503), but no follow-up work has appeared in the literature. Another early spectral survey examined the e.p.r. of crude *Clostridium* nitrogenase and compared particles from $N_2$-grown *Azotobacter* with particles from $NH_4^+$-grown *Azotobacter* (520); the study indicated that e.p.r. analysis could have utility in nitrogenase investigations, but the spectra revealed little information beyond the suggestion of $N_2$-induced spectral effects and an indication of Mo, Fe and Mn in the preparations, as has recently also been found in e.p.r. studies of nitrogenase from lupine and soybean bacteroids (553).

More recent e.p.r. analyses of intact *Azotobacter* nitrogenase show its spectrum to be qualitatively identical to that of Mo-Fe protein (102). Purified nitrogenase preparations have resonances at g-values of about 4.3, 3.7 and 2.0, and on reduction with dithionite, also at a g-value of 1.94. The resonance at g=3.7 has been detected in whole cell samples of several diazotrophs (p. 77).

The e.p.r. method has been used to examine inhibitor effects and metal function in *Azotobacter* preparations (336, 337), but the data must be interpreted with caution because of the impurity and low biological activity of the samples used. Reagents such as $N_2$, CO, and diazobenzene-sulfoxylate were proposed to react preferentially at a $N_2$-activating center. These gave a weak signal in the regions of g-values of 1.94 and 2.01 and were interpreted to mean interaction with Fe (336, 337); in another report, $N_2$ was observed to cause the signal with g-value of 1.94 to disappear (455). Nitric oxide, which blocked electron transport, produced a transformation in the signal from 1.94 and 2.01 to a new signal at a g-value of 2.03, characteristic of iron nitrosyl complexes associated with cysteine ligands; *p*-chloromercuribenzoate inhibited $H_2$ evolution activity, but did not affect the e.p.r. spectrum in one report (336), while it eliminated the resonance at a g-value of 1.94, as did *o*-phenanthroline, in another (337). Preparations from cells grown on $^{57}Fe$ showed line broadening at a g-value of 1.94, but not 2.01 (337). No spectral lines attributable to Mo were readily discernible in the functional enzyme preparations, but on denaturation with acid or $O_2$ a resonance at a g-value of 1.98 assigned to Mo became apparent; from measurements of line width and relative increase in resonance intensity with denaturation, it was estimated that the distance between Mo and another paramagnetic center, indicated to be iron, is 6-7 Å (337).

Mössbauer spectra have been obtained for whole cells of $N_2$-grown *C. pasteurianum* and *A. vinelandii* (335) and for the reconstituted nitrogenase of *A. vinelandii* (706) and *K. pneumoniae* (371). In the intact *Azotobacter* studies the relative amount of cellular Fe in the $2^+$ state was observed to increase in $N_2$ and to decrease in Ar. The Mössbauer spectrum of reconstituted *A. vinelandii* nitrogenase was simply the sum of its components' spectra. This applies also to the *Klebsiella* nitrogenase spectrum except for a weak signal reported to be unique for the mixture of Mo-Fe protein, Fe protein, ATP and dithionite (371); however, the validity of the *Klebsiella* spectra from this study is placed in question by the more recent data with purer material (200).

The visible spectrum of nitrogenase preparations has received little attention, though the failure of CO to alter the spectrum was interpreted in one report as evidence that Fe did not participate in $N_2$ binding (677).

## C. Stability of Nitrogenase

As indicated in the previous discussions of the Mo-Fe and Fe proteins, the most conspicuous sensitivities of nitrogenase are associated with exposure to low temperature and to $O_2$. Some parallels exist between the cold and the $O_2$ sensitivities in that nitrogenases of the more anaerobic organisms seem to be more vulnerable, and an increase in vulnerability with purification is characteristic of both sensitivities. In addition the Fe protein is uniquely involved in the cold inactivation, and it is likewise the more $O_2$ sensitive component.

Purified *Azotobacter* nitrogenase is quite stable at room temperature and at pH values near neutrality, provided that it is rigorously protected from exposure to $O_2$. Preparations stored under such conditions show only slight loss of activity after as long as six weeks, during which time increasing amounts of black precipitate may form, but do not influence activity (105). Crude nitrogenases from other sources, for example, soybean bacteroids (386) and *Clostridium* (96), are not as stable at room temperature as the *Azotobacter* enzyme, due possibly to the action of contaminating hydrolytic enzymes in the preparations; generally, however, they are more stable at $22^{o}$-$25^{o}$ than at or near $0^{o}$ (see below). The nitrogenases of *Azotobacter* (98, 288), *Clostridium* (195, 463) and *Bacillus* (727) are reasonably stable to heating as shown by retention of activity after $50^{o}$-$60^{o}$ treatment for 10-15 min, while soybean nitrogenase is destroyed by such treatment (742).

*1. $O_2$ Sensitivity*

Oxygen affects nitrogenase in two ways: 1. reversible uncompetitive inhibition of ATP hydrolysis and substrate reduction (p. 120), and 2. inactivation of the enzyme, which is generally considered to be irreversible, though reactivation of specifically the Fe protein of soybean module bacteroids has been reported (691, 751). The sensitivity to $O_2$-inactivation requires that strictly anaerobic conditions be maintained with most nitrogenase preparations. While both protein components of nitrogenase are sensitive to $O_2$, the Fe protein is by far the more labile. The degree of sensitivity to $O_2$ increases with $pO_2$ and with temperature, as might be expected, and also varies among the different organisms and methods of cell rupture and/or purity (170, 369, 736). Thus, nitrogenases of the more aerobic $N_2$-fixing bacteria are generally less sensitive to $O_2$-inactivation, and among these the cruder preparations are more resistant than the more highly purified. For example, crude extracts of *Azotobacter* nitrogenase prepared with a French press are quite insensitive to $O_2$ up to the purification step with protamine sulfate. In a comparison of crude nitrogenase preparations the order of resistance to $O_2$ inactivation was *Azotobacter>Klebsiella>Bacillus>Clostridium* with the range extending from no loss of activity with *Azotobacter* to 75% loss with *Clostridium* after exposure to air for 10 min at $20^{o}$ (369). In apparent contradiction to this order the nitrogenases of *Mycobacterium* (57) and of the blue-green algae *Plectonema* and *Anabaena* (300, 636) are similar in sensitivity to the nitrogenases of the more anaerobic bacteria.

The effect of various additives on $O_2$-inactivation of nitrogenase *in vitro* has been examined. The extent of $O_2$ damage to the nitrogenases of *Mycobacterium* (59) and *Azotobacter* (736) was greatly mitigated by cellular particulate material. *Azotobacter* nitrogenase also responds to additions of soluble extract components, with NADH dehydrogenase protecting against $O_2$, and cytochrome $c_4$, azotoflavin, ferredoxin or a dialyzable extract factor enhancing sensitivity (736). Interaction of these latter components with nitrogenase was indicated by their capacity to stimulate the catalytic activity of nitrogenase when the enzyme was present at low concentrations and to inhibit at higher nitrogenase levels (736). Reducible substrates, including $N_2$ and $C_2H_2$, failed to protect against $O_2$-inactivation (369), while thiols and $Na_2S_2O_4$ protected, apparently by scavenging $O_2$ from the incubation mixture (57, 195, 222). Additions of ATP increased the rate of $O_2$-inactivation of *Mycobacterium* nitrogenase, and this led to the speculation that ATP transformed nitrogenase into an active form characterized by an increased exposure of $O_2$-sensitive sites (59).

The mechanism of $O_2$-inactivation is obscure, but the spectral changes, both e.p.r. and visible (pp. 88, 92), that accompany $O_2$ exposure offer obvious leads for further work, and the ability to restore inactivated bacteroid Fe protein with $Fe^{2+}$ and reductants may be pertinent (p. 96) Dialysis of an $O_2$-bleached preparation of *Klebsiella* Mo-Fe protein did not remove Mo or Fe (369), while loss of Mo was observed on aerobic dialysis of *A. vinelandii* Mo-Fe protein (105). Attempts to quantitate the interaction between $O_2$ and *Azotobacter* Mo-Fe protein indicated that only one or two $O_2$ molecules were sufficient to inactivate each Mo-Fe protein.

In whole cells, sensitivity to $O_2$ can be demonstrated with all free-living diazotrophs. Among the facultative and aerobic bacterial diazotrophs this sensitivity is clearly greater when the organisms are cultured on $N_2$, which presumably reflects the $O_2$-sensitivity of the $N_2$-fixing system. This is well known for the facultative species, which fix $N_2$ best or only under anaerobic conditions; it has long been known for *Azotobacter* (see Ref. 194), and has more recently been demonstrated with the other aerobic species, *Derxia* (306) and *Mycobacterium* (58). This distinction, however, may not apply to the blue-green algae. Although these diazotrophs fix $N_2$ more actively at subatmospheric $pO_2$, recent analyses of *Anabaena* and *Nostoc* indicate that some other component may be more $O_2$-labile than nitrogenase (667). As indicated above, $N_2$ fixation by free-living aerobic diazotrophs is generally inhibited even at $pO_2$ levels below 0.2 atm; however, it is possible to adapt *Azotobacter* to grow on $N_2$ at partial pressures of $O_2$ extending up to 0.6 atm (194).

The effects of $O_2$ on *A. chroococcum* have been intensively studied by POSTGATE and co-workers (147, 148, 193, 194, 307). To explain the relative immunity of the $N_2$-fixing system of *Azotobacter* to $O_2$, they have proposed the concept of two protective mechanisms for this organism. One is described as "conformational protection" and the other as "respiratory protection". The former contends that when nitrogenase is challenged by $O_2$ *in vivo*, it changes shape, reversibly converting to an $O_2$-insensitive, but catalytically inactive species; this concept is based on $C_2H_2$ reduction data which show that *Azotobacter* nitrogenase activity can rapidly and completely recover from short-term exposures to inhibitory levels of $O_2$; significantly, inhibitors of protein synthesis did not affect recovery rates. Respiratory protection refers to a proposed relationship between nitrogenase and the well-known $O_2$-stimulated respiratory rate of *Azotobacter*. The concept holds that the increased rate of $O_2$ consumption is a protective mechanism in which incoming $O_2$ is intercepted by respiratory components and utilized for carbon oxidation. Accordingly, at higher levels of $O_2$ considerable quantities of carbohydrates are consumed to maintain this defense, and the efficiency of $N_2$ fixation, in terms of mg $N_2$ fixed per gm carbon source consumed, suffers drastically.

Respiratory protection of $O_2$-sensitive enzymes has been described for microorganisms other than *Azotobacter* (7), but the extent to which it or conformational protection may apply to other diazotrophs is open to question. Recent studies with *Anabaena* do not support these mechanisms for that organism, and therefore possibly for blue-green algae in general. LEX et al. (423) concluded that the $O_2$-stimulated increase in *Anabaena* respiration in fact contributed to inhibition of nitrogenase activity rather than to its protection; specifically, competition for reductant was implicated, with the algal photorespiratory system gaining competitive advantage at higher $pO_2$ levels. BONE (72) observed that recovery of *Anabaena* from exposure to high $pO_2$ was slow and, moreover, was blocked by numerous inhibitors of

protein synthesis. The alga thus gave no indication of possessing the conformational change protective mechanism indicated for the aerobic bacterium; rather, it appeared that $O_2$ caused irreversible loss of nitrogenase, which could then be replaced only by synthesis of new enzyme.

Since the blue-green algae normally photoevolve $O_2$, it is likely that these diazotrophs have developed specific mechanisms to protect nitrogenase from $O_2$ of metabolic origin. STEWART has suggested that a type of compartmentalization is involved in which nitrogenase is localized in the heterocyst cells (p. 23) under aerobic conditions, but also develops in the vegetative cells as the environment becomes increasingly anaerobic (659). The concept has received experimental support from studies with *Anabaena* (252) and is consistent with the physiological differences between the two types of algal cells. The heterocysts lack photosystem II and so do not photoevolve $O_2$, and they show evidence of a reductive interior even under aerobic conditions. The heterocyst environment thus appears well suited for nitrogenase regardless of external $pO_2$. The vegetative cells on the other hand photoevolve $O_2$ and would be expected to develop and sustain nitrogenase only at a relatively low $pO_2$, since the combined effects of internal $O_2$ production and high external $pO_2$ would probably block nitrogenase biosynthesis and/or function.

Tests of $O_2$ effects on $N_2$ fixation by excised soybean nodules show this symbiosis to be more resistant to $O_2$-inactivation than any other diazotroph tested. Optimum $^{15}N_2$ fixation by nodule slices occurred at 0.5 atm $O_2$ and significant activity was evident at 0.8 atm (112). The possible protective effect of leghemoglobin must be considered here; however, protection via facilitated diffusion of $O_2$ (the probable function of leghemoglobin; p. 164) seems inadequate to explain the high resistance of nodule *slices* to $O_2$, though the explanation is attractive for intact nodules. Of course, additional protective mechanisms may be operative, including specialized membranes such as the vesicles surrounding bacteroids. In studies with intact soybean nodule bacteroids, which require $O_2$ (for ATP synthesis) to fix $N_2$, it was shown that as the $pO_2$ was increased, the $K_m$ and $V_{max}$ of $N_2$ reduction increased in approximate proportion; the increased $V_{max}$ was attributed to increased ATP availability, but the nature of inhibition causing the change in $K_m$ could not be established (51).

### *2. Cold Lability*

A loss of activity at temperatures around $0^{\circ}$ appears to be generally characteristic of nitrogenases, though the extent of sensitivity to cold varies with the source organism, method of extraction, degree of purity, and environmental conditions. Storage at extremely low temperature is not detrimental (386, 451, 492, 647). The Fe protein is the sole susceptible part of the enzyme, with the Mo-Fe protein showing no negative response to low temperature exposure.

Crude preparations of *Azotobacter* nitrogenase are stable to cold, but on purification these become sensitive (93); however, a high purity particulate preparation was stable at $4^{\circ}$ (329), and $MgCl_2$ at 5 m*M* was reported to effectively protect purified (about 95%) *Azotobacter* nitrogenase from inactivation at $0^{\circ}$ for up to one month (623). Cytochrome (including mammalian) or catalase provided protection against cold inactivation when added to preparations prior to chilling, and furthermore, these proteins even restored substantial activity to cold-inactivation preparations (370). *Clostridium* extracts likewise

lose activity rapidly in the cold (96, 195, 196, 726); the rate of inactivation was much slower at pH 6.7 than at higher or lower pH (195). Crude cold-inactivated preparations containing hydrogenase could be activated by incubation under $H_2$ at room temperature (195). Addition of lipids did not protect against the cold, but some restorative effects of reducing and sulfhydryl agents were noted (196). Extracts of *Anabaena* lost about 60% of their activity after 12 hrs at $0^{\circ}$ (300). Crude *Bacillus* extracts lost 25% of initial activity at $-10^{\circ}$ in 48 hrs, but lost even more at $4^{\circ}$ (726).

In contrast crude extracts of *Chromatium* (721) and *Gloeocapsa* (242) did not exhibit cold sensitivity. Additionally, soybean bacteroid extracts have been reported to be more stable at $0^{\circ}$ than at $25^{\circ}$ (386), though a conflicting report indicates the contrary (51) and cold sensitivity has been shown for excised nodules (498). However, the most striking exception to the general rule of cold lability appears to be the Fe protein of *Klebsiella* , which is insensitive even in the pure state (200).

## D. Integrity of Reconstituted Nitrogenases

It is not yet clear whether nitrogenase formed by recombination of its two fractions is identical in all respects to the unfractionated enzyme. Qualitatively, there appears to be no difference, but quantitative differences have been noted. For example, reconstituted *Clostridium* nitrogenase possesses a much more vigorous reductant-independent ATP hydrolysis activity (p. 116) than does the unfractionated enzyme (88, 342, 723); similarly, this activity is about twice as great in reconstituted *Azotobacter* nitrogenase as in the native enzyme, accounting for about one sixth of total ATP hydrolysis (92, 269). Another apparent anomaly with *Azotobacter* preparations is the great range in the ATP:2 electron ratio (p. 114) found for reconstituted nitrogenase. Values of from 2.1 to 13.2 are reported (368) in contrast to more commonly found values of 4 to 5 obtained with native *Azotobacter* nitrogenase (92, 269).

It is possible that damage to one or the other nitrogenase protein may cause some of the anomalous behavior observed, though this has not been examined in detail. If this is a factor, it is likely that the reconstituted nitrogenases would frequently show damage effects, since the component proteins are normally subjected to more hazards through handling; additionally, on fractionation they lose whatever stabilizing effect may be afforded by mutual interaction. It is clear that preparations of homogeneous, unfractionated nitrogenase are essential for definition of nitrogenase composition and characteristics. At this time no such preparations are available.

# Nitrogenase: The Catalysis

A casual examination of the nitrogenase reaction scheme (Fig. 4-5) shows that it describes two relatively distinct functions, one involving the sequence

$$\text{ATP} + \text{reductant} \xrightarrow[\text{Active sites for reductant and ATP}]{M^{2+}} \text{ADP} + P_i + e^-$$

and the other involving a reaction with $N_2$ or a similar reducible substrate, as

$$N_2 + e^- + H_3O^+ \xrightarrow[\text{Active site for reducible substrate}]{} NH_3$$

In the following discussion the first function is treated under the heading "Nitrogenase Turnover" and the second under "Substrate Reduction". The data on nitrogenase catalysis show that these two functions can be treated separately without distorting the concept of a unified reaction. Enzyme turnover is in fact absolutely independent of substrate reduction since $H_3O^+$ can oxidize the reductant/ATP site(s), presumably without the participation of the reducible substrate site. On the other hand, substrate reduction is dependent on the turnover function, but only in the sense that turnover maintains a flow of reductant; this link between the two functions is considered under the heading "Electron Allocation". The characteristics of the reducible substrate binding site(s), binding kinetics and other aspects of substrate reduction are otherwise independent of enzyme turnover.

## I. Nitrogenase Turnover

The numerous compounds and reaction conditions that influence the rate of nitrogenase turnover are discussed in this section. These include the nature and concentration of all necessary reactants, as well as the effects of such external factors as pH, temperature and inhibitors. Of the four reactants required for nitrogenase turnover - ATP, $M^{2+}$, $H_3O^+$ and reductant - only reductant appears to affect reaction rate in a direct manner that conforms to simple Michaelis-Menten kinetics. More complex rate effects are caused by variations in ATP concentration and by enzyme concentration or composition. These complex effects are examined in detail in the following two sections. The key to understanding nitrogenase turnover may well rest in defining the peculiarities and unique aspects of these two reaction parameters. Effects of the divalent cation are discussed in conjunction with ATP. The utilization of dithionite as an electron donor to nitrogenase is duscussed here, while the various physiological nitrogenase reducing agents and systems are described in Chapter 8.

## A. Enzyme Concentration and Composition

Even in early studies with nitrogenase preparations it was apparent that the relationship between enzyme concentration and activity was neither direct nor simple. More specifically a so-called "dilution effect" was often observed in plots of nitrogenase activity vs. nitrogenase concentration. The effect produces curves that are sigmoidal, or concave upward, becoming linear at high enzyme concentration, but with the linearity failing to extrapolate through the origin (91, 242, 285, 289, 488, 623, 642) (Fig. 6-1). The sigmoidal character of enzyme-activity relationship has been interpreted as evidence that nitrogenase exists as an active complex in equilibrium with its dissociated,

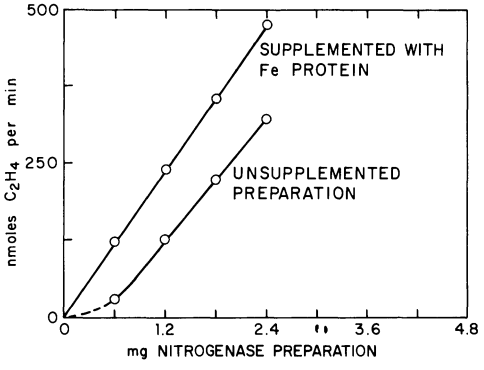



Fig. 6-1. The "dilution effect" in nitrogenase activity measurements and its correction by additional Fe protein (104)

inactive components, but as was discussed previously, direct evidence for this has not been reported. The "dilution effect" has been overcome in some cases with additional Fe protein (610, 643). In other cases supplementation with additional Mo-Fe protein was reported to overcome the effect (343, 610), but there is conflicting evidence for this (643). Such supplements as a dialyzable extract factor, ferredoxin, flavodoxin, cytochrome and NADH dehydrogenase (736) overcame the effect, but boiled extract (98) or bovine albumin (98, 643, 723) did not, nor did extract of nitrogenase-repressed cells or Mo-Fe protein (in preparations relieved by Fe protein) (643). Dilution of nitrogenase prior to assay did not influence the effect, indicating that inactivation of nitrogenase by dilution in the reaction mixture was not a contributing factor (723). Because the "dilution effect" causes specific activity to vary with protein concentration, it has been proposed that specific activities be derived from a curve as in Figure 6-1 and expressed as

$$\frac{\text{Activity (in standard units)}}{p^2 - x^2}$$

where p, the amount of extract protein assayed, and x, the amount of protein at the point of extrapolation, are squared because of sigmoidicity (643); a linear relationship between activity and protein was indicated for the data in Ref. 643 by application of this expression. Additionally, the "dilution effect" can be produced with preparations which do not naturally exhibit it by reducing the concentration of ATP or by adding salts as detailed below.

Activity profiles obtained by titrating one nitrogenase protein against the other (p. 81) generally have shown a hyperbolic relationship between activity and concentration of the varied protein (51, 52, 499, 689), but sigmoidicity has been observed on occasion with *Clostridium* and soybean Fe protein (53, 343, 501). Hill plots (log v/V-v *vs.* log mg Fe protein) of the reactions which showed sigmoidicity were observed to have slopes of about 2, which was interpreted to mean that two Fe proteins participated in the reaction sequence. The addition of excess Mo-Fe protein, but not Fe-protein, has been shown to be inhibitory (52, 386, 433, 501, 610). This inhibition suggests that more than one Fe protein must complex with each Mo-Fe protein to form an effective catalytic unit, and that with excess Mo-Fe protein the Fe protein is bound primarily in the ineffective or less effective complexes of one Fe protein per Mo-Fe proteins. The sigmoidal titration curves mentioned above support this concept. Alternatively, it is possible that in an effective complex a Mo-Fe protein and an Fe protein must attach at more than one point, and that with excessive Mo-Fe protein the attachment sites on Fe proteins become occupied by two Mo-Fe proteins, each of which prevents the other from completing an effective complexation. The situation is analogous to that indicated in classical substrate inhibition. Recombination studies with the *Clostridium* proteins showed that excess Mo-Fe protein specifically inhibited electron transfer and did not affect ATP hydrolysis (433). High levels of $O_2$-inactivated Fe protein did not inhibit the nitrogenase activity of mixtures of Mo-Fe protein and Fe protein except at low ATP levels, where the effect was attributed to the inactive protein trapping ATP (56). It thus appears that $O_2$-inactivated Fe protein may not complex with Mo-Fe protein; however, Fe protein of low activity was indicated to inhibit Fe protein of high activity (751).

Additional complexity in the nitrogenase concentration-activity relationship is implicit in reports which show that different activities of nitrogenase are catalyzed optimally by different ratios of Mo-Fe protein:Fe protein. These data were discussed in Chapter 5 (p. 99). A differential effect on activities was also noted in cold-treated *A. vinelandii* preparations, which lost $N_2$- and $C_2H_2$-reducing activity more rapidly than cyanide-reducing activity (370). Other effects observed with recombined nitrogenase systems may be relevant to the nitrogenase concentration-activity relationship. These include the high level of reductant-independent ATP hydrolysis (p. 116) found in recombined fractions of *Clostridium* nitrogenase (486), the wide variation in the ratio of ATP hydrolyzed:electrons transferred observed with reconstituted nitrogenases (368, 433) and effect of Fe protein concentration on the $K_m$ values of reducible substrates (p. 96).

## B. ATP

It is likely that the unique character of nitrogenase function is intimately associated with the role(s) of ATP, yet this substrate remains one of the most puzzling parts of the nitrogenase story. Unfortunately, no good theoretical basis for the ATP requirement of nitrogenase is apparent. Though the partial reduction of $N_2$ to diazene is highly exergonic, the over-all reduction to $NH_3$ by, for example, $H_2$ is thermodynamically favored (13), as shown in the equation,

$$N_2 + 3H_2 \rightleftarrows 2NH_3\ (g)\ \Delta F^{o} = -7.95\ \text{kcal/mole}\ (25^{o}),$$

and it is difficult to rationalize the requirement on the basis of energy needs. The lack of a theoretical basis for ATP involvement in

nitrogenase-catalyzed reduction of $N_2$ to $NH_3$ has undoubtedly exerted a long-standing negative effect on research in this field. Without theoretical support early workers were deprived of a stimulus and guide which could have accelerated the discovery that ATP was in fact required for activity, and even today research would surely benefit from the ideas and experimentation generated by a sound theory. Elsewhere in enzymology there appears to be no useful analogy that might help clarify ATP function in nitrogenase activity. Accordingly, conclusions concerning an ATP role must come almost exclusively from the specific data obtained with nitrogenase reactions, although some recent model experiments may be relevant. The bulk of the enzyme data relates either to the effect of ATP concentration on reaction rates, or to the relationship between ATP hydrolysis and electron transfer. These aspects of ATP participation in nitrogenase turnover will be considered in turn below.

It should perhaps first be pointed out that in certain respects ATP hydrolysis by nitrogenase is not at all unusual. Like various other ATP-hydrolyzing enzymes, nitrogenase displays absolute specificity for ATP and shows no activity with GTP, CTP, or UTP (285). Hydrolysis products are ADP and $P_i$ (288, 376). The divalent cation requirement is not atypical and may reflect a need for an ATP-metal complex, as concluded in one report (376). However, since the nitrogenases of numerous diazotrophs show optimum activity with an ATP:$M^{2+}$ ratio of 2, it is likely that free ATP may be an important species, perhaps in addition to the ATP-metal complex. The nitrogenase of *Azotobacter* (97), *Clostridium* (500), *Chromatium* (721), *Klebsiella* (540) and lupine (374) show such an optimum when supported by substrate levels of ATP; on the other hand optimum ratios of 1 have been reported for *Clostridium* (88) and 0.67-1.0 for *Anabaena* (301), and the lupine enzyme is almost as active with a ratio of 0.5 as with 2.0 (374). When ATP is supplied at a constant low level by means of an ATP-generating system, the ATP:$M^{2+}$ ratio does not appear to be highly critical, but an optimum ratio of less than 1 is indicated (98, 498). Other divalent cations, including $Mn^{2+}$, $Co^{2+}$, $Fe^{2+}$, and $Ni^{2+}$, can replace $Mg^{2+}$ in the order of effectiveness indicated, but $Mg^{2+}$ gives best results (97, 177, 288, 301, 540). Cuprous ion and $Zn^{2+}$ are inhibitory (100% at 0.001 *M*), and $Ca^{2+}$ is either inhibitory (288) or has no effect (97). Nitrogenase is normally assayed with the aid of an ATP-generating system because of the inhibition resulting from high levels of ATP or from the ADP which accumulates in the absence of a generating system. The creatine kinase/creatine phosphate generating system is most commonly used, and $Mg^{2+}$ adequately meets the divalent cation requirements of both nitrogenase and creatine kinase.

*1. ATP and Reaction Rate*

a) Relationship of ATP, Enzyme Concentration and Activity

Involvement of ATP in the relationship between enzyme concentration and activity is apparent from the ATP saturation curves obtained for high and low concentrations of *Azotobacter* enzyme (103, 104) (Fig. 6-2). At the low nitrogenase concentration a sigmoidal curve was obtained but with more enzyme present the response was essentially hyperbolic. Additional complexity was indicated for the ATP saturation curves obtained with lupine nitrogenase (374). These plots showed a plateau region in the 2-4 m*M* ATP range and were considered indicative of more than one role for ATP. It should be borne in mind, however, that the substrate used in the lupine experimentation was cyanide, and the

nature of the nitrogenase complex required for cyanide reduction may be different than for $N_2$ reduction (p. 99). As a consequence of the dependency of ATP saturation kinetics on nitrogenase concentration, an apparent dilution effect is observed for reactions conducted at low ATP concentration (104, 623).

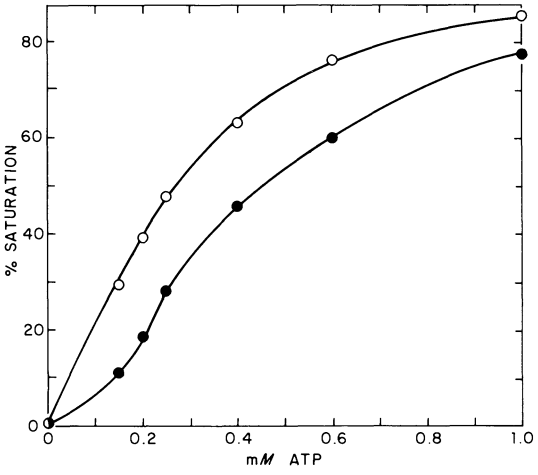



Fig. 6-2. Saturation of nitrogenase with ATP, showing effect of nitrogenase concentation. ●, 0.6 mg purified nitrogenase per ml; ○, 2.4 mg per ml. Activities obtained at 5 m*M* ATP were taken as 100% (104)

Hill plots of log v/(V-v) *vs.* log ATP concentration have been used to estimate the number of ATP molecules involved in the rate-limiting step of the nitrogenase reaction, though their applicability to nitrogenase catalysis has been questioned (374) on the basis that the plateau character of the ATP saturation curve means that the mechanism of nitrogenase catalysis does not involve the concerted transition state for which the Hill equation was developed. In cases where Hill plots are applicable, the value of the slope is a direct measure of the "interaction coefficient", which is correlated with the number of reactive sites responding to the varied substrate; ideally, whole integer values indicate the number of independent sites and non-integer values indicate positive or negative cooperativity among sites. The plots constructed from the curves showing ATP saturation of nitrogenase yield slopes with values of about one for *Klebsiella* (540) and two for *Clostridium* (500) and *Azotobacter* (623), suggesting the involvement of one or two molecules of ATP in the rate-limiting step; multiphasic slopes were found for the Hill plots of the lupine and soybean nodule nitrogenase saturation curves. The lupine curve had a slope near 1 in a plateau area and near 2 on either side, suggesting mixed positive and negative cooperativity (374). The soybean curves had slopes near 1 at low ATP concentration, but at higher ATP levels these increased to about 2 for $N_2$ reduction by 0.25 mg nitrogenase and to about 5 for $C_2H_2$ reduction by 1.65 mg nitrogenase (53).

b) Michaelis Constant

Values for the $K_m$ of ATP have appeared in the nitrogenase literature, but it appears now that the complexity of ATP kinetics renders these

values meaningless as kinetic constants and useful only in an operational sense. This complexitiy is further emphasized by the sigmoidal rather than linear nature of the double reciprocal plots, $v^{-1}$ *vs.*$(ATP)^{-1}$, for nitrogenase (53, 374). A linear relationship does hold, however, between $v^{-1}$ and $(ATP)^{-2}$ (104). At high levels of protein and ATP the deviation from linearity in the $v^{-1}$ *vs.*$(ATP)^{-1}$ plots is less apparent; linearity has been sufficiently good to permit evaluation of the apparent $K_m$ for ATP from such plots, and values of from 0.12-0.3 m*M* ATP have been reported (56, 97, 242). From linear plots of $v^{-1}$ *vs.* $(ATP)^{-2}$ values for $K_m$ calculated according to case (3) (below) are 0.2-0.5 (104) and 0.7 m*M*[9].

DIXON and WEBB (178) discuss two general cases applicable to such kinetics. Both arise from reaction sequences which utilize two identical substrate molecules. The first involves enzymes which catalyze reactions that are second order in a single substrate. The other involves reactions in which a substrate can also function as an activator. In both cases, treatment according to Michaelis-Menten theory leads to equations in which $v^{-1} = V^{-1} + CS^{-1} + C'S^{-2}$ where C and C' are constants or combinations of constants, v is velocity, V is maximum velocity and S is the substrate concentration. Plots of $v^{-1}$ *vs.*$S^{-1}$ are linear or nearly so at high substrate concentrations where the $S^{-2}$ term is negligible. At low substrate concentrations, however, the $S^{-1}$ term becomes negligible, and the shape of the curve is determined by $S^{-2}$. Under the latter conditions the plot of $v^{-1}$ *vs.*$S^{-1}$ is sigmoidal, but if the data are plotted as $v^{-1}$ *vs.*$S^{-2}$, a linear response is obtained. Some cases in which these kinetics can apply are discussed below, based on the general treatment described in Ref. 178. The first three to be considered are variations of reactions involving two substrates, with the specification that the two substrates are identical; the fourth case involves activation of the enzyme by the substrate.

1. Where the two substrate molecules attach randomly at two different sites with different affinities, and the addition of the second substrate molecule is influenced by the prior attachment of the first, the following reactions apply:

$$\begin{aligned} EE' + S &\rightleftharpoons EE'S \\ EE' + S &\rightleftharpoons SEE' \\ EE'S + S &\rightleftharpoons SEE'S \\ SEE' + S &\rightleftharpoons SEE'S \end{aligned}$$

where EE' is enzyme, E and E' are binding sites for S, the substrate. The corresponding equilibrium equations are:

$$\begin{aligned} (e - p_a - p_b - p)s &= K_a p_a \\ (e - p_a - p_b - p)s &= K_b p_b \\ p_a s &= K_b' p \\ p_b s &= K_a' p \end{aligned}$$

where e, s, $p_a$, $p_b$ and p are the concentrations of total enzyme, substrate, EE'S, SEE' and SEE'S. Velocity, v, will be proportional to p and maximum velocity, V, will be proportional to e:

$$v = kp \qquad V = ke$$

---

[9] The value of 0.7 m*M* was determined from the straight line obtained by replotting the graph of $v^{-1}$ *vs.*$(ATP)^{-1}$ (Fig. 2b in Ref. 374) as $v^{-1}$ *vs.* $(ATP)^{-2}$.

Combining the equilibrium equations gives

$$v = \frac{ke}{1 + \frac{K'_a}{s} + \frac{K'_b}{s} + \frac{K_a K'_b}{s^2}}$$

or $$v = \frac{V}{1 + \frac{K'_a}{s} + \frac{K'_b}{s} + \frac{K_a K'_b}{s^2}}$$

or $$\frac{1}{v} = \frac{1}{V} + \frac{1}{V}\left(\frac{K'_a}{s} + \frac{K'_b}{s}\right) + \frac{1}{V}\left(\frac{K_a K'_b}{s^2}\right)$$

When s is very small $$\frac{1}{v} = \frac{1}{V} + \left(\frac{K_a K'_b}{V}\right)\frac{1}{s^2}$$

The plot of $\frac{1}{v}$ *vs.* $\frac{1}{s^2}$ should be linear, and when $\frac{1}{v} = 0$,

$$\frac{1}{s^2} = -\frac{1}{K_a K'_b}$$

The intercept of the **x**-axis is then the negative reciprocal of the product of the dissociation constant of EE'S (or SEE') times the constant for the dissociation of SEE'S to EE'S + S (or SEE' + S). Specific variations of 1. are considered in 2. and 3.

2. Where the binding of the first substrate has no effect on the binding of the second and s is very small, then $K_a = K'_a$, and $K_b = K'_b$, and the intercept of the x-axis becomes $-\frac{1}{K_a K_b}$, or the negative reciprocal of the product of the dissociation constants associated with each of the two sites.

3. If the further restriction is made that the identical substrate molecules combine with identical affinity, then $K_a = K'_a = K_b = K'_b = K_s$, and the intercept of the **x**-axis becomes $-\frac{1}{K_s^2}$.

4. In the case where the substrate can serve as an activator the applicable reactions are

$$E + S \rightleftharpoons ES$$
$$E + S \rightleftharpoons ES_a$$
$$ES_a + S \rightleftharpoons ES_aS$$

where $S_a$ is the substrate combined as activator; the reactions shown and their subsequent development are based on the assumptions that ES is inactive or nearly so and that S and $S_a$ bind at separate sites and do not compete with each other.

The equilibrium equations for the above reactions are, respectively,

$$(e-p-q-r)s = K_s q$$
$$(e-p-q-r)s = K_{sa} r$$
$$rs = K'_s p$$

where q, r and p are the concentrations of ES, $ES_a$ and $ES_aS$, respectively, and $K_s$, $K_{sa}$ and $K'_s$ are the respective dissociation constants. Velocity will be proportional to p, so that

$$v = \frac{ke}{1 + \frac{K'_s}{S}\left(1 + \frac{K_{sa}}{K_s} + \frac{K_{sa}}{S}\right)}$$

or $$\frac{1}{v} = \frac{1}{V} + \frac{K'_s}{V}\left(1 + \frac{K_{sa}}{K_s}\right)\left(\frac{1}{s}\right) + \left(\frac{K_{sa}K'_s}{V}\right)\left(\frac{1}{s^2}\right).$$

When s is very small,

$$\frac{1}{v} = \frac{1}{V} + \left(\frac{K_{sa}K'_s}{V}\right)\frac{1}{s^2}$$

and the plot of $\frac{1}{v}$ *vs.* $\frac{1}{s^2}$ will be linear.

At $\frac{1}{v} = 0$, $\frac{1}{s^2} = -\frac{1}{K_{sa}K'_s}$, that is, the negative reciprocal of the product of the dissociation constants for the activator-enzyme complex and the activated enzyme-substrate complex.

Present data do not distinguish which of the above cases, or of their numerous possible variations, may be applicable to nitrogenase. Their relevancy is considered in the discussion of nitrogenase mechanism (Chapter 7).

c) ADP and Salt Inhibition

Inhibition of nitrogenase activity by ADP has long been known, but the nature of inhibition has not been established. Its effect on the ATP saturation curve is to cause or greatly increase the sigmoidal nature of the curve, indicating direct competition at ATP binding sites (87, 374, 500). With lupine nitrogenase the ADP inhibition curve contained intermediary plateau regions (374), and with both lupine and *Clostridium* nitrogenase ADP caused Hill coefficients for ATP to change from about two to about three; these effects were interpreted as evidence that ADP functions as a negative modifier in a manner inverse to the activation by ATP (374, 500). Lupine and *Klebsiella* nitrogenases appear to be particularly sensitive to ADP, showing in the case of lupine 50% inhibition at about one tenth the ATP concentration used and in the case of *Klebsiella* about 90% inhibition when ADP concentration was one half that of ATP; with *Clostridium* (376) and *Azotobacter* (368), ADP at about half the ATP concentration gave 50% inhibition. In one report (540) ADP was described as a competitive inhibitor of $C_2H_2$ reduction, an ambiguous description of what the data show to be competitive inhibition of ATP (in spite of an apparent misprint in the legend of Fig. 4 of Ref. 540). Apparent inhibition of nitrogenase activity by AMP has been observed, but has been attributed to ADP derived from AMP via the action of contaminating adenylate kinase (500).

Reaction rates are severely affected by high levels of salts, which bring on sigmoidicity in ATP saturation kinetics and also cause a dilution effect in the activity-nitrogenase concentration relationship (104; Fig. 6-3). Inhibition of *Azotobacter* nitrogenase by NaCl, KCl, $KNO_3$, $NH_4Cl$, LiCl, $K_2SO_4$ cacodylate and phosphate salts has been demonstrated (98, 104, 285, 631, 642); with the exception of one report (440), no unique effects are observed with ammonium salts. The linear relationship between $v^{-1}$ and $(ATP)^{-2}$ is maintained under conditions of salt inhibition, but $V_{max}$ is decreased and the $K_m$ for ATP

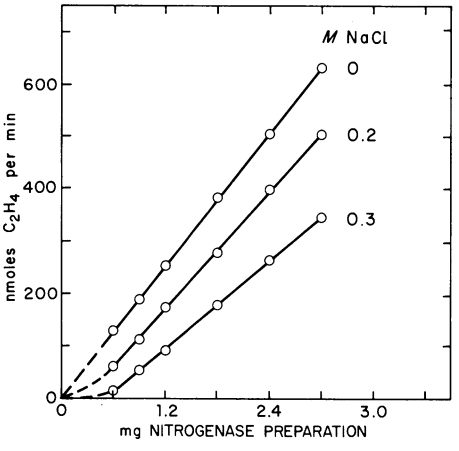



Fig. 6-3. "Dilution effect" caused by salt (104)

is increased (104). The reciprocal plot suggests noncompetitive inhibition of the hyperbolic type described by MAHLER and CORDES (449) in which stable inhibitor-enzyme-substrate complexes, but no inhibitor-enzyme complexes are formed. However, plots of $v^{-1}$ *vs.* inhibitor (i) did not produce the common x-axis intercept expected for noncompetitive inhibition, and it is likely that the inhibition is mixed (104). The additional type of inhibition is indicated to be competitive by the nature of the kinetic plots. A point of interest in the relationship between reaction rate, v, and salt concentration, (i), is that plots of $v^{-1}$ *vs.* $(i)^2$ were linear, whereas plots of $v^{-1}$ *vs.* (i) were not, indicating bimolecular salt function reminiscent of the apparent bimolecularity of the ATP function. Large concentrations of ATP did not reverse the salt inhibition appreciably, nor did they produce a line parallel to the x-axis in the plot of $v^{-1}$ *vs.* $(i)^2$, as would occur if inhibition were purely competitive.

*2. The ATP:2-Electron Ratio*

The question of the stoichiometric relationship between the molecules of ATP hydrolyzed and the number of pairs of electrons transferred by nitrogenase to an acceptor has received considerable attention, but produced no definitive answer. Observed stoichiometries are usually expressed in terms of the ATP:$2e^-$ ratio, where "ATP" is the difference between the amount of ATP hydrolyzed in the presence *vs.* the absence of reductant, and "$2e^-$" is the number of electron pairs ac-

counted for in reduction products. The ATP value thus presumably excludes the contribution from reductant-independent ATP hydrolysis activity, as discussed previously. Although it is clear that considerable quantities of ATP are hydrolyzed, there is at present no compelling reason to believe that a precise, rigid stoichiometry exists or has any mechanistic significance for nitrogenase catalysis (433, 498, 630). It is apparent that the relative amounts of electrons transferred and ATP hydrolyzed, as well as the mode(s) of hydrolysis, are pertinent aspects of the catalytic sequence that somehow couples these two activities. Values ranging from less than 2 (630) to 20 (433) have been reported for ATP:$2e^-$. The ratio can be consistent for a given set of conditions, but has been reported to vary with the ratio of Mo-Fe protein to Fe protein, temperature, pH, ADP concentration, reducible substrate and prior history of the preparation.

In a recent study the effect of the *A. vinelandii* Mo-Fe protein:Fe protein ratio on the ATP:$2e^-$ ratio was examined. As the relative amount of Mo-Fe protein was increased the ATP:$2e^-$ ratio also increased; values of from 4 to more than 20 could be obtained by combining appropriate amounts of the two proteins (433). In work with fractions of *A. chroococcum* nitrogenase, however, the opposite effect was observed (368); with Fe protein held constant a fourfold increase in Mo-Fe protein caused the ATP:$2e^-$ ratio to decrease by as much as 50%; the decrease was not consistent, however, and in other reactions the variation in Mo-Fe protein caused no change in ATP:$2e^-$. Of possible relevance to the effect of protein balance on ATP:$2e^-$ is the indication that heterologous recombinations of Mo-Fe protein and Fe protein produced higher ATP:$2e^-$ ratios than did homologous recombinations (369). A variation in ATP:$2e^-$ with the history of a particular preparation is implicit in the wide range of values found in the literature, and is in fact documented in one report (630) in which a preparation that was subjected to drastic treatments during purification gave a higher ratio than preparations treated more gently.

In the temperature range 20-40° the ATP:$2e^-$ ratio increased from about four to about six; the effect of temperature on reductant-independent ATP hydrolysis was not given, but relatively high corrections accounting for at least 23% of ATP hydrolysis were made for this activity (269). An increase in total ATP hydrolyzed by reconstituted *Clostridium* nitrogenase, as pH was decreased from 7.6 to 5.6, was attributed to variation in reductant-independent ATP hydrolysis activity, and after corrections for the high levels (33% or more) of this activity, a constant value of 2 ATP:$2e^-$ was obtained over the pH range (342). In unfractionated *Clostridium* nitrogenase the ratio increased with increasing pH with a value of near 4.0 at pH 6.0 and near 4.6 at pH 8.0 (723). In contrast no variation from a ratio of 5 was observed for *Azotobacter* nitrogenase over the pH range 6-8 after correcting for reductant-independent ATP hydrolysis, which almost doubled as pH was decreased from 8 to 6 (269).

Other factors reported to influence the ATP:$2e^-$ ratio include ADP and the type of reducible substrate present. In one report the ATP:$2e^-$ ratio was found to decrease in the presence of ADP; it is unclear whether this effect was caused by ADP inhibition of reductant-independent ATP hydrolysis or for some other reason (630). Variation attributable to the type of reducible substrate used has been reported from comparisons of the amount of $H_2$ evolved under Ar *vs.* the amounts of $C_2H_4$ + $H_2$ formed in the presence of $C_2H_2$; higher ratios were found for the reaction under Ar, where $H_2$ is the sole reduction product; ratios of 3.5 for $H_2$ *vs.* 2-3 for $C_2H_4$ + $H_2$ were determined in one report (368) and 4.3 *vs.* 2.7 in another (329). In other work, no differ-

ence has been observed in such a comparison (451). In the comparison which gave 2-3 for $C_2H_4$ + $H_2$, ratios of up to 13 were found for methyl isocyanide (368). No effect on the ratio has been observed with dinitrogen (relative to no added substrate) and the equivalency, [moles $H_2$ + 3/2 moles $NH_3$ formed in a reaction run under $N_2$] = [moles $H_2$ formed in a companion reaction run under inert gas], appears to hold consistently (269, 376, 623, 723).

Whether the ATP:$2e^-$ values observed with nitrogenase *in vitro* have any physiological significance is debatable. Studies of $N_2$-fixing *A. chroococcum* cells indicated that only 4 or 5 moles ATP were consumed per mole $N_2$ fixed under low $pO_2$ (148). This is far more efficient than the value of 15-20 moles ATP per $N_2$ typically found for nitrogenase preparations and suggests that the coupling of ATP hydrolysis to electron transfer is seriously disrupted *in vitro*. In contrast to the low ratio determined for intact *Azotobacter* cells, however, are the values of 20 moles ATP per $N_2$ fixed determined for *Clostridium* cells (142), and about 30 for *Klebsiella* (565).

In summary, it is increasingly apparent that the ATP:$2e^-$ ratios, or stoichiometry, that are observed simply reflect the degree of uncoupling, or non-productive ATP hydrolysis, of a particular preparation or assay condition; if this is the case, it is pointless to consider observed ATP hydrolysis values as mechanistically significant constants. However, it is not unreasonable to assume that a stoichiometry exists between electron transfer and productive ATP hydrolysis, and that this relationship might be evaluated if non-productive ATP hydrolysis were eliminated or could be adequately corrected for. In all likelihood, productive ATP hydrolysis is the norm *in vivo*, and uncoupling an artifact of extraction.

### *3. Reductant-Independent ATP Hydrolysis*

A factor which must be reckoned with in the analysis of ATP data is the hydrolysis of ATP which occurs in the absence of reductant. This is particularly critical in evaluating the ATP:$2e^-$ ratio. The extent and significance of the "reductant-independent ATPase" activity is unclear and the question of how to treat this activity in the interpretation of nitrogenase activity has always been confusing. Like the reductant-dependent hydrolysis of ATP, which is associated with effective electron transfer to substrates or $H_3O^+$, the reductant-independent reaction requires both proteins of nitrogenase and $Mg^{2+}$ (342). The activity can be measured only in the absence of reductant, and thus any effects, either inhibitory or stimulatory, by reductant on this activity remain unknown and uncorrected for. In estimations of its contribution to total ATP hydrolysis under reducing conditions (i.e., under normal nitrogenase assay conditions), it is assumed that the activity is unaffected by reductant and can be evaluated directly from a "minus-reductant" control reaction; however, the validity of this assumption has been challenged (368, 433). The reaction was initially presumed to be caused by contaminating ATPases present in nitrogenase preparations, but the requirement for both nitrogenase proteins makes it unlikely that this is the case.

In unfractionated nitrogenase preparations the reductant-independent activity was calculated to account for about 15% of total ATP hydrolysis as measured both with *Azotobacter* (92, 269) and with *Clostridium* (723). The reaction has been examined in detail using *Clostridium* nitrogenase reconstituted from its Mo-Fe protein and Fe protein components (88, 342). With the reconstituted nitrogenase the reductant-

independent activity accounted for about 80% of total ATP hydrolysis at pH 6.4 (Fig. 3 in Ref. 88), and in a subsequent study (342) it accounted for about one third of the total ATP hydrolysis at the pH of 6.6 used for nitrogenase activity assay. The total ATP hydrolysis did not vary significantly over the pH range 5.2-7.6, but as pH was decreased below 6.5, reductant-independent hydrolysis activity accounted for an increasing fraction of the total until at pH 5.2 it was responsible for all ATP hydrolysis. Similarly, with *Azotobacter* preparations the reductant-independent activity increased as pH decreased (269). The activity in *Clostridium* preparations was shown to be inhibited by ADP (88) (which is known to inhibit normal nitrogenase activity as measured by substrate reduction and thus presumably is inhibiting reductant-dependent ATP hydrolysis), and it was catalyzed at optimal rates by a reconstituted nitrogenase preparation more highly enriched in Mo-Fe protein than the preparation which was optimal for substrate reduction (88). Excessive levels of *Azotobacter* Mo-Fe protein were also implicated in causing high ATP:$2e^-$ ratios (433).

### *4. Other ATP Effects*

The concentration of ATP influenced the allocation of electrons (p. 121) during $N_2$ reduction by *Azotobacter* nitrogenase so that the $N_2$ reduced: $H_2$ evolved ratio increased as the concentration of ATP increased (623). In other *Azotobacter* work no ATP effect on electron allocation was indicated during $C_2H_2$ reduction (105). If ATP concentration can influence electron allocation during cyanide reduction, then the ATP saturation curves reported for lupine nitrogenase (374) must be re-evaluated, since activities were assumed to relate directly to the amounts of methane formed from cyanide.

Variations in $K_m$ values of *Azotobacter* nitrogenase for $N_2$ and $C_2H_2$ have been correlated with ATP concentration (53, 103), with decreases of about 50% observed as ATP was decreased from a saturating level to about 0.25 m*M*. These results suggest that the decrease in $K_m$ for $N_2$ previously observed with soybean nodule nitrogenase at low $pO_2$ (44, 51) might also have been due to a decrease in ATP concentration, since $O_2$ is presumably responsible for providing ATP in the nodule system.

## C. Dithionite

Dithionite (hydrosulfite, $S_2O_4^{2-}$) is the most widely used reductant for nitrogenase assays *in vitro*. It is the only known non-physiological electron donor that can be used in substrate amounts with nitrogenase, and it appears to have universal application among nitrogenase preparations. It acts directly on the enzyme and requires no intermediate carrier. The only other non-physiological agents known to reduce nitrogenase directly are certain viologen dyes, but these have application only in catalytic amounts as carriers for coupling some physiological reductants to nitrogenase.

The $K_m$ for dithionite has been determined with the nitrogenases of several diazotrophs, and values ranging from less than $10^{-6}M$ to $10^{-2}M$ have been reported. These are listed in Table 6-1. The wide range in values is difficult to reconcile. Though the reagent functions effectively with all nitrogenases tested, inhibitory effects also have been observed (242, 635), possibly due to impurities or degradation products (below).

Table 6-1. Michaelis constants reported for dithionite

| Source of nitrogenase | $K_m$ ($M \times 10^6$) | Analytical method | Reference |
|---|---|---|---|
| *Gloeocapsa* | 10,000 | unspecified | 242 |
| *A. vinelandii* | 9,000 | $H_2$ evolution | 97 |
| *A. vinelandii* | 8,000 | $N_2$ reduction, ATP hydrolysis | 328 |
| Soybean bacteroids | 800 | unspecified | 394 |
| *C. pasteuranium* | < 10 | Spectrophotometric assay of dithionite disappearance | 432 |

The particular feature(s) which make dithionite so effective as reductant for nitrogenase are not clear, nor are the reasons for the ineffectiveness of other reductants; the low reduction potential of dithionite is considered a necessary characteristic, though this obviously is not the unique property of the reagent for nitrogenase reduction. Dithionite can reduce both one- and two-electron acceptors, and in its reaction with nitrogenase one mole of dithionite is consumed per pair of electrons used by the enzyme (433, 621). The significance of these reduction characteristics is not clear, since the enzyme can couple with one-electron carriers, such as the viologen dyes and *Anabaena* ferredoxin (p. 152) as well as two-electron carriers, such as the bacterial ferredoxins and flavodoxins, though these two carriers may function as single-electron transfer agents.

In practice dithionite solutions are usually prepared from $Na_2S_2O_4$ which is available commercially at about 85-90% purity. Bisulfite and thiosulfate are reported to be common impurities (63, 442), but their effects on nitrogenase have not been determined. However, it appears that harmful impurities and/or degradation products are sometimes encountered with commercial dithionite. Best results are usually obtained with reagent that has been stored in absolute dryness and is dissolved in $O_2$-free water or buffer shortly before use; in anaerobic solution at $0^{\circ}$ and pH above 7, the reagent is stable for about one day. It reacts readily with $O_2$, generating a variety of products which include bisulfite, bisulfate, $H_3^+O$ and others. Among these appear to be products harmful to nitrogenase. In contrast the (unidentified) oxidized products formed from dithionite during the reduction of nitrogenase do not appear to affect nitrogenase activity. Solutions of dithionite are colorless, but they absorb strongly at 315 nm, and this feature is the basis for an assay procedure described in Chapter 1.

## D. External Factors

In addition to the obvious ability of enzyme, reductant, ATP and divalent cation to affect nitrogenase turnover, other factors can influence reaction rate. These include the conditions of pH and temperature and also the action of various inhibitors. The effects of such imposed, or external, factors are discussed below.

### *1. pH*

Most nitrogenase show maximal activity near pH 7 but have a broad optimum; for example, the optimum pH and pH range for activity of *Azotobacter* and *Clostridium* nitrogenase are similar with the optimum for *Azotobacter* somewhat above pH 7 and for *Clostridium* slightly below. In

both cases activity decreases gradually with about 90% remaining at ±0.5 pH unit from the optimum (96, 177, 241, 269, 342). Extracts of *Anabaena* show a broad pH optimum between pH 7 and 8 (301, 636); *Gloeocapsa* nitrogenase activity declines sharply below pH 7.0 but more gradually above (242).

In practice the nitrogenase reaction is frequently supported by an ATP-generating system, which prevents $H_3O^+$ accumulation and eliminates the need for buffering; in fact unbuffered reaction mixtures were reported to be superior to buffered reaction mixtures, probably because the buffer increases ionic strength to an inhibitory level (p. 113). In most instances buffers are used whether needed or not. The most common include *Tris*-HCl, TES and particularly with extracts of the photosynthetic organsims, HEPES. A comparison of buffers showed TES to be slightly superior to other buffers tested (269). Phosphate and cacodylate, formerly used extensively, are now seldom employed, the former because of its inhibitory action and the latter because of the health hazards resulting from arsine formation.

*2. Temperature*

Nitrogenase shows two noteworthy responses to temperature, both of which may be involved in control, especially at low temperatures. One, the cold lability of the enzyme, has been described (p. 104). In additon, a biphasic effect of temperature on reaction rate is observed, though one report with *Azotobacter* extracts does not show this (427). Biphasicity has been demonstrated with *Azotobacter* nitrogenase and with whole *Clostridium* cells (98, 285). Arrhenius plots show that a change in temperature dependency occurs at about 20°, but that the dependency is linear on either side of this critical temperature. The reaction is not affected adversely up to at least 40° (269). Activation energy values of about 14 kcal/mole above 20° and 35 kcal/mole below 20° have been calculated for *A. vinelandii* nitrogenase reactions. These values are independent of the nature of reducible substrate, which shows that activation of the electron acceptor is not rate limiting for nitrogenase activity. An apparent increase in the ATP:$2e^-$ ratio with temperature indicates that the activation energies of ATP hydrolysis and electron transfer may differ slightly (269). This is not consistent with other work, including the report that shows linear Arrhenius plots (427) in which values of 19.2, 20, and 21.4 kcal/mole were obtained for $N_2$ reduction, ATP hydrolysis and $H_2$ evolution, respectively.

Biphasic Arrhenius plots such as the one observed with nitrogenase have been reported for more than twenty enzymes. Numerous explanations for this phenomenon have been proposed, but most are difficult to test experimentally. An early theory held that temperature-dependent shifts from one controlling "master reaction" to another were responsible, but this concept has been broadly criticized on theoretical grounds (see Ref. 178 for discussion). Explanation based on temperature-dependent conformational changes between two stable forms, both enzymically active but differing in activation energy, have been proposed (178) and are supported experimentally (454). A refinement, or special case of this concept, has been described in which the "temperature breaks" are attributed to phase changes that occur at the critical temperature (402). This explanation is based on the assumption that the enzyme can exist in two discrete states or phases, each with a characteristic activity constant; only at the critical temperature for phase transition can the phases be in equilibrium and when the system deviates from this temperature, conversion to exclusively one

phase occurs. Whether any of these explanations is relevant to nitrogenase remains to be seen. The multi-protein nature of nitrogenase and the multi-substrate nature of the nitrogenase reaction suggest a particularly complex catalysis dependent on many association-dissociation phenomena, and temperature effects on these equilibria are not yet known. The existence of at least two functional states of nitrogenase is implicit in the allosteric description of the enzyme (p. 133), but the influence of temperature on transitions between states has not been examined.

*3. Inhibitors*

Only agents that inhibit the turnover rate of nitrogenase are considered here. Salts in high concentrations and ADP may be valid candidates in this context, but inhibition by these agents has already been discussed (p. 113). Inhibition of reducible substrates is discussed in the following section on substrate reduction. It is generally assumed that the inhibitors described below affect both ATP hydrolysis and electron transfer, but this point is not well established in many cases. The early literature on nitrogenase inhibition is covered in detail in a chapter by BRADBEER and WILSON (78).

WONG and BURRIS (729) have established that $O_2$ uncompetitively inhibits substrate reduction and ATP hydrolysis catalyzed by the nitrogenase of subcellular *Azotobacter* particles. The $K_i$ values for $O_2$ varied among the substrates tested, with 0.014, 0.023, 0.008, 0.003 and 0.025 atm found for $N_2$, $C_2H_2$, azide, cyanide and ATP, respectively. At high levels of $O_2$, nitrogenase is irreversibly inactivated.

Metal binding agents including α,α-dipyridyl, Tiron (1, 2-dihydroxybenzene-3,5-disulfonate) and *o*-phenanthroline inhibited *Azotobacter* nitrogenase by more than 50% at $10^{-3}M$, but were relatively ineffective at $10^{-4}M$ (93, 288, 455); 2,3-dimercaptopropanol inhibited only 17% at $10^{-3}M$ (93). *Anabaena* nitrogenase showed greater sensitivity to metal binding agents (301), but *Clostridium* nitrogenase appeared to be considerably more resistant, showing no inhibition by α,α-dipyridyl or Tiron at $10^{-3}M$ (376). Nitrogenases of all three organisms were inhibited by sulfhydryl reagents, including mersalyl *(Clostridium)*, *p*-chloromercuribenzoate, *p*-chloromercurisulfonate *(Azotobacter* and *Anabaena)* and iodoacetate *(Azotobacter)*. The latter was indicated to inhibit only Fe protein (677).

Heavy metal inhibition has been observed with *Azotobacter* nitrogenase, which was completely inhibited by $Cu^{2+}$ and $Zn^{2+}$ at $10^{-3}M$ (97); mixed results have been reported for $Ca^{2+}$ (97, 288). Other reagents showing inhibition include NADH, glutathione (288) and diazobenzenesulfonic acid (336). Phosphorylation uncouplers and electron transport inhibitors are generally ineffective, but a few show activity at high concentrations, including $10^{-2}M$ arsenate (288, 462), $10^{-3}M$ dinitrophenol (336) and $10^{-3}M$ CCCP (*m*-Cl-carbonylcyanide phenylhydrazone). Fluoride does not specifically inhibit nitrogenase and was used to inhibit pyrophosphatase activity in experiments which established $P_i$ as the hydrolysis product of ATP in nitrogenase catalysis (288). Inhibition by diazene, hydrazine and hydroxylamine has been shown with *Clostridium* extracts (115, 243).

Most of the inhibitors described previously have not been examined in depth with the nitrogenase system, and except for $O_2$ inhibition, the types of inhibition involved are unknown. In view of the apparent importance of metals and sulfide in nitrogenase, the observed inhibition

patterns are not unexpected, while the absence of inhibition activity for uncouplers or inhibitors of electron transport is somewhat surprising and mechanistically disappointing, but indicates that the role of ATP in electron transfer by nitrogenase is different from that of energy-coupled electron transfer by mitochondria.

## II. Substrate Reduction

Up to this point in the discussion of nitrogenase the fate of electrons activated by the enzyme has received little attention, though it has been emphasized that as far as enzyme turnover is concerned, where the electrons go does not really matter. In a broader sense, of course, the fate of these electrons is of utmost significance. Pecularities of enzyme turnover notwithstanding, the ability of nitrogenase to reduce triple-bond substrates remains its most unique property. The characteristics of these reductions are examined in the present section, following a preliminary discussion of electron allocation in nitrogenase reactivity.

### A. Electron Allocation: $H_3O^+$ *vs.* Exogenous Reducible Substrate

As indicated previously the rate of nitrogenase turnover, measured as rate of total electron transfer, depends on the degree to which the enzyme is saturated with ATP and reductant. The rate is independent of exogenous electron acceptors, since $H_3O^+$ can function in this role, giving $H_2$; pH and $D_2O$ studies indicate that $H_3O^+$ is not limiting in the pH range of nitrogenase activity (339). Electron transfer to $H_3O^+$ at the expense of transfer to an added reducible substrate is a universal characteristic of nitrogenase activity *in vitro*. It is not known whether transfer to $H_3O^+$ is a major physiological reaction; it could be an artifact resulting from, for example, greater exposure of a reduced site on acellular nitrogenase to attack by $H_3O^+$. The $H_2$-evolving site does not appear to be the same as the substrate reduction site(s), since CO does not interfere with $H_2$ evolution, though it potently inhibits the reduction of exogenous substrates (Fig. 4-5). Unfortunately, there are no known agents that interfere with $H_2$ evolution that do not also interfere with the reduction of exogenous substrates. $H_2$ evolution is unaffected by $pH_2$ (98).

The mechanism that governs allocation of electrons to either $H_3O^+$ or added reducible substrates (or the condition that renders nitrogenase vulnerable to interference by $H_3O^+$) is unknown. The allocation can be measured with reasonable ease in most cases by comparing the amounts of $H_2$ produced in reaction mixtures which are identical except that one contains an added reducible substrate. The fraction of activated electrons utilized for reduction of added substrate is here defined as the electron allocation coefficient:

$$\text{electron allocation coefficient} = \frac{\text{electrons to exogenous reducible substrate}}{\text{electrons to } H_3O^+ + \text{exogenous reducible substrate}}$$

This coefficient varies with the nature and concentration of reducible substrate, the character of nitrogenase, the $D_2O$ concentration for certain nitriles and perhaps the concentration of ATP. These factors are discussed below.

Electron allocation is affected by the concentration of added reducible substrate, as would be expected, and normal saturation curves are described for all nitrogenase substrates tested. What is not entirely expected is the observation that these substrates (with the possible exception of $C_2H_2$), even at saturating levels do not use the full electron flux generated by nitrogenase (see p. 106), and consequently their reduction is always accompanied by some $H_2$ evolution. The electron allocation coefficients of saturating levels of added substrates vary over a wide range, from almost 1.00 for $C_2H_2$ to less than 0.01 for some of the nitriles. In general, the higher the $K_m$ value for a substrate, the lower the electron allocation coefficient. For example, with $N_2$ as substrate about 75% of the electrons are utilized in $N_2$ reduction, with the remaining 25% going into $H_2$ evolution for an electron allocation coefficient of 0.75; this coefficient is observed at 1 atm $N_2$, which is more than 5 times the $K_m$ of about 0.18 atm, as well as at 6 atm $N_2$ (269).

Variations in the character of nitrogenase appear to affect electron allocation. The most striking example of this is seen in a comparison of electron allocation by V-nitrogenase (p. 85) *vs.* Mo-nitrogenase. With V-nitrogenase a considerably higher proportion of electrons was utilized for $H_2$ evolution at the expense of $N_2$ or $C_2H_2$ reduction (100). Though the $K_m$ value of $C_2H_2$ was significantly different in the V-nitrogenase reactions, saturating levels of the substrates were used, and it is apparent that the electron allocation effect was not simply an indirect effect due to lack of saturation. In comparisons of reconstituted nitrogenase containing a high ratio of Mo-Fe protein to Fe protein, a greater proportion of electrons was observed to be utilized for $H_2$ evolution *vs.* reduction of $C_2H_2$ or methyl, ethyl, or vinyl isocyanide (368); no $K_m$ values were determined. An effect of ATP on electron allocation was indicated by *Azotobacter* nitrogenase assays in which the product ratio $NH_3$:$H_2$ decreased when ATP concentration was decreased from 0.1 m*M* to 0.025 m*M* (623).

A unique feature of the reduction of substituted nitriles is a characteristic 2- to 5-fold increase in electron allocation coefficient when the reduction is carried out in $D_2O$ rather than $H_2O$ (241). As judged from the reduction of acrylonitrile, the $D_2O$ effect appears to be exerted directly on electron allocation, since $D_2O$ did not alter the $K_m$ of nitrogenase for acrylonitrile. These alterations in electron allocation were attributed to probable similarities in the activation energies of nitriles and $H_3O^+$ and $D_3O^+$ (see p. 129). Other effects on electron allocation observed in acrylonitrile reduction included a diminished allocation to acrylonitrile at high ionic strength or at pH values above and below 7.0; these variables may have affected acrylonitrile binding, which was not tested for each condition. At levels of acrylonitrile high enough to cause substrate inhibition, allocation to acrylonitrile decreased with the time of incubation.

## B. Specificity and Inhibition

Nitrogenase occupies a unique niche in the spectrum of enzyme specificity as the only isolated enzyme known to reduce the triple bond. Furthermore, it can in fact reduce no substrate that lacks a triple bond or potential triple bond, and in this respect it differs from all other defined enzymes, which deal only with single and/or double bonds. Within the limits of this specificity nitrogenase exhibits considerable variation in the nature of its substrates and the character of its reductions. Susceptible triple bonds include only, but not all, those which bridge N to N, N to O, N to C and C to C; in

all known examples at least one of these atoms is terminal or bonded to H.

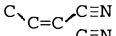
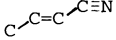
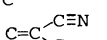
The reducible substrates are conveniently categorized into 6 different classes: $N_2$, azide, $N_2O$, nitriles, isonitriles and alkynes. When these are listed in sequence of decreasing N≡N character to increasing C≡C character, an order is apparent in the nature of the reduction products from nitrogenase catalysis (Table 6-2). At one extreme, N≡N is completely reduced in a reaction which consumes 6 electrons, cleaves the molecule and generates no detectable free intermediates, though it is feasible to consider that stable enzyme bound intermediates - diazene and hydrazine - are formed in the course of reduction. At the other extreme is HC≡CH. This is potentially a 6-electron acceptor, but it is reduced only as far as ethylene. Between the two extremes, the R-C≡N and R-N≡C substrates show variable reactivity which includes the generation of multiple reduction products from the same substrate and reaches a high degree of complexity in the reductive condensation sequence observed with isonitriles.

The substrates of classes 1-5 can be considered as R≡X compounds in that the triple bond is terminal in all cases. Reduction of these substrates results in cleavage of the triple bond. For substrates of classes 1-4 with terminal N, $NH_3$ is produced, but if O is terminal, $H_2O$ (presumably) is produced. For class 5 substrates (isonitriles), however, the product from the terminal C is not limited to methane. Alkyl and alkenyl hydrocarbons containing up to at least 3 carbons are formed by a condensation mechanism in which alkyl-nitrogenase intermediates are probably involved (see p. 130). Class 6 compounds (alkynes) possess a terminal ≡CH (or, as with allene, a potential terminal ≡CH) which is reduced only by $2e^-$ to the corresponding alkene.

A particularly interesting substrate is cyanide, which has the R≡X character of a typical class 1-4 substrate, the ≡CH character of a class 6 substrate and even the -N≡C character of class 5, since at reaction pH its major form is $N{\equiv}C^-$ rather than HCN. The products of cyanide reduction indicate that it does in fact function as a multiclass substrate. It has not been shown to yield $CH_2{=}NH$, as expected from class 6 behavior, but may yield $CH_3NH_2$ and $CH_4 + NH_3$, as expected from class 1-4 behavior, and possibly traces of ethane and ethylene, as expected from class 5 behavior. The relative amounts of product formed indicate that the dominant behavior is as a class 1-4 substrate, yielding primarily $CH_4 + NH_3$.

Carbon monoxide can well be considered as the classical inhibitor of nitrogenase reductions. It reversibly inhibits the reduction of all known electron acceptors except $H_3O^+$. Dinitrogen reduction and $C_2H_2$ reduction were inhibited to about the same extent by CO with $K_i$ values found in the range $6\text{-}40 \times 10^{-3}$ atm for these substrates (100, 285, 435). The fragmentary data for other substrates suggest that these compete more successfully with CO. For example, azide reduction was inhibited only 50% by $16 \times 10^{-3}$ atm CO, a level which almost totally inhibited $N_2$ reduction in a comparative experiment (599); at 0.2 atm CO, $N_2O$ reduction still proceeded at almost 10% of the uninhibited rate (287). The $K_i$ for CO inhibition of $CH_3NC$ reduction was reported at 8 or $80 \times 10^{-3}$ atm (based on the two values given in Ref. 366, $6.8 \times 10^{-5} M$ and $6.8 \times 10^{-6} M$); yet elsewhere 0.4 atm CO was reported to inhibit by only about 50% (368). Most analyses indicate CO to be a competitive inhibitor (285, 366, 435), though it was reported also to inhibit noncompetitively (329), and competitive inhibition was not consistently demonstrable in at least one case (100). Carbon monoxide does not affect ATP hydrolysis or $H_2$ evolution even at 1.0 atm. It is indicated to specifically affect Mo-Fe protein (677).

Table 6-2. Reducible substrates of nitrogenase

| Reduction characteristics | Substrates | | Products | $K_m$ (m*M*) | Electron allocation coefficient |
|---|---|---|---|---|---|
| Exhaustive reduction ($6e^-$); no intermediates | 1. Dinitrogen | $N\equiv N$ | $NH_3 + NH_3$ | 0.1 | 0.75 |
| Partial reduction ($2e^-$); reductive bond cleavage | 2. $HN_3$ or $N_3^-$ | $^{2-}N\text{-}^{+}N\equiv N$ | $N_2 + NH_3$ | 1 | 0.70 |
| | 3. Nitrous Oxide | $^{2-}N\text{-}^{+}N\equiv O^{+}$ | $N_2 + H_2O$ | 1 | 0.70 |
| | 4. Nitriles | $RC\equiv N$ | $C_nH_{2n}$, $C_nH_{2n+2}$ + $NH_3$ or $RNH_2$ | | |
| | Cyanide | $HC\equiv N$, $CN^-$ | n=2 n=1,2 + $NH_3$ or $CH_3NH_2$ | 0.4 to 1.0 | 0.35 |
| | Unsaturated | | | | |
| Reductive cleavage of N to $NH_3$; partial and complete reduction of R group; electron allocation enhanced by $D_2O$; ($4\text{-}8e^-$) | Acrylo- | $C\text{=}C\text{-}C\equiv N$ | n=3 n=3 + $NH_3$ | 10 to 50 | 0.20 |
| | *cis*-Crotono- |  | n=4 n=4 + $NH_3$ | 100 to 200 | 0.0005 |
| | *trans*-Crotono- |  | n=4 n=4 + $NH_3$ | | 0.0005 |
| | Methacrylo= |  | n=4 + $NH_3$ | | 0.0002 |
| | 3-Butene- | $C\text{=}C\text{-}C\text{-}C\equiv N$ | n=4 n=4 + $NH_3$ | | 0.002 |
| | Saturated | | | | |
| | Aceto- | $C\text{-}C\equiv N$ | n=2 + $NH_3$ | > 500 | < 0.01 |
| | Propio- | $C\text{-}C\text{-}C\equiv N$ | n=3 + $NH_3$ | > 500 | < 0.01 |
| | Butyro- | $C\text{-}C\text{-}C\text{-}C\equiv N$ | n=4 + $NH_3$ | | < 0.001 |
| | 5. Isonitriles | $RN\equiv C$ | $C_nH_{2n}$, $C_nH_{2n+2}$ + Amines | | |
| Reductive cleavage of $-N\equiv C$ bond to yield amines and C-1 addition products, including both alkenes and alkanes up to at least 3C length. ($6\text{-}14e^-$) | Methyl- | $C\text{-}N\equiv C$ | n=2,3 n=1,2,3 | 0.2 to 1 | 0.60 |
| | Ethyl- | $C\text{-}C\text{-}N\equiv C$ | n=2 n=1,2 | 10 to 25 | 0.25 |
| | Vinyl- | $C\text{=}C\text{-}N\equiv C$ | n=2 n=1,2 | 0.8 | 0.25 |

6. Alkynes

| Partial reduction only ($2e^-$) | | | | | |
|---|---|---|---|---|---|
| Acetylene | C≡C | C=C | | 0.1 to 0.3 | 1.00 |
| Propyne | C-C≡C | C-C=C | | | |
| Butyne | C-C-C≡C | C-C-C=C | | | |
| Allene | C=C=C | C-C=C | 0.43[a] | | 0.72 |

[a] atmospheres

Nitric oxide is a potent inhibitor of $N_2$ reduction and was shown to inhibit competitively with a $K_i$ of $25 \times 10^{-3}$ atm ($4.3 \times 10^{-7} M$), similar to that of CO (435). Inhibition of $CH_3NC$ reduction by NO was also reported, but this substrate appeared to be much less sensitive, with 0.15 atm NO required for about 50% inhibition (368). Its effect on $H_2$ evolution and ATP hydrolysis has not been tested, but it can be concluded from the $CH_3NC$ work that this aspect of nitrogenase activity is little affected or unaffected by NO.

Inhibition of $N_2$ reduction by $H_2$ has been intensively studied since it was first found that $H_2$ could not serve as a diluent gas in studies of the apparent $K_m$ of $N_2$ in whole plants (78, 669, 717). This inhibitor appears to possess the unique property of specifically inhibiting $N_2$ reduction (623), though two reports indicate that the reductions of $CH_3NC$ and $C_2H_2$ are also inhibited (366, 624); however, the inability of $H_2$ to inhibit $C_2H_2$ reduction has been confirmed (105), and the reductions of acrylonitrile and propionitrile were unaffected by $H_2$ (241). The reported inhibition by $H_2$ of substrates other than $N_2$ may be due to impurities in the $H_2$ used. As mentioned earlier (p. 121), $H_2$ does not inhibit ATP-dependent $H_2$ evolution.

In contrast to CO and NO, $H_2$ is a relatively ineffective inhibitor of $N_2$ reduction, and $K_i$ values are reported in the range 0.2-0.5 atm (269, 339, 366, 394, 435). Like CO and NO, $H_2$ appears to be a competitive inhibitor. An early inhibition study with crude *Clostridium* extracts showed non-competitive inhibition by $H_2$ at high phosphate concentrations (435), but this phosphate effect may occur in the phosphoroclastic reaction, not nitrogenase, since it was not observed with purified *Azotobacter* nitrogenase (105).

As might be expected, each reducible substrate inhibits the reduction of other reducible substrates. Most data suggest that these inhibitions are competitive, but non-competitive inhibition has also been observed. In one extensive analysis, the type of inhibition exerted by CO, $H_2$ and the various reducible substrates was found to be competitive for some substrates and non-competitive for others (329). The data were interpreted to favor the existence of five sites, or modified sites: 1. an $H_2$, $N_2$ site, 2. a $C_2H_2$ site, 3. a HCN, $HN_3$, $CH_3NC$ site, 4. a CO site, and 5. an $H_3O^+$ site. Assignments were made to the same site on the basis of competitive inhibition and to different sites on the basis of non-competitive inhibition. Since non-competitive inhibition does not preclude interaction of the inhibitor at the substrate site (449), it is possible that sites 1-4 are one and the same. In other kinetic studies (53, 540), Hill

plots of log v/V-v *vs.* log concentration of $N_2$, $C_2H_2$, HCN or $HN_3$ produced slopes of one, indicating a single binding site per nitrogenase molecule; however, the data do not distinguish whether the same or different sites were involved in each case. It is clear that additional studies are required to clarify these intersubstrate inhibitions.

## C. The Reducible Substrates

### *1. $N_2$*

Dinitrogen is the physiological substrate of nitrogenase and until 1966 was the only established reducible substrate of the enzyme. Though the alternate substrates described below will no doubt continue to aid in the clarification of the catalytic mechanism of nitrogenase, the physiological significance of the enzyme must be defined within the context of $N_2$ reduction.

The reduction of $N_2$ exhibits three phenomena not observed in the reduction of other substrates: 1. it is inhibited by $H_2$, 2. it is accompanied by an exchange between the protons of $H_2$ and the protons of water and 3. its $K_m$ is the same for both V-nitrogenase and Mo-nitrogenase. The common involvement of $H_2$ suggests (1) and (2) may be two ways of looking at the same phenomenon, and this is in harmony with a proposed mechanism (339) in which $H_2$ can interact with the $N_2$-occupied, as well as the unoccupied, $N_2$-binding site (see Fig. 8-3). In the latter case competitive inhibition of $N_2$-binding results; in the former $H_2$ can interact with the H of bound intermediates of $N_2$ reduction, which have their origin in $H_2O$.

The $K_m$ values found for $N_2$ *in vitro* usually fall in the range 0.05-0.20 atm or 0.03-0.12 m*M* (272, 290), though values outside this range have been reported (for example, Refs. 53, 104). Increases in $K_m$ values were correlated with increases in Fe protein and ATP concentration (53, 104). In general $K_m$ values determined *in vitro* are significantly greater than the values accepted for growing cultures, (for example, *A. vinelandii* and *Nostoc* cultures, 0.02 atm; *Clostridium* cultures 0.03 atm; see Ref. 110). At saturation *(in vitro)* about 75% of activated electrons are utilized for $N_2$ reduction. Both CO and NO are strong and apparently competitive inhibitors, while $H_2$ is a weak competitive inhibitor with similar affinity as $N_2$ for the common binding site. All other reducible substrates inhibit, but the nature of inhibition is not well defined.

It is evident that the only products of $N_2$ reduction are two molecules of ammonia formed by the addition of 6 protons and electrons to $N_2$. No free intermediates have been detected, in spite of numerous efforts to establish their presence (110); the extensive attempts to detect such candidates as diazene and hydrazine led to the conclusion that free intermediates are not formed. However, circumstantial evidence strongly favors the formation of enzyme-bound transients, specifically diazene and hydrazine, generated in the course of sequential electron addition to the bound $N_2$. This evidence includes 1. the inability of nitrogenase to catalyze $^{15}N_2 \rightleftharpoons {}^{14}N_2$ exchange, which argues against an initial N≡N bond cleavage, as presumably occurs in heterogenous catalysis (272), 2. the nature of nitrile and isonitrile reduction products, which form analog series based on increments of two electrons, as well as azide, nitrous oxide and alkyne reductions which involve only two electrons, and 3. the similarity between the $D_2 + H_2O \rightleftarrows HD + HDO$ exchange reactions of nitrogenase and of a nitrogenase model (546). This exchange occurs only when $N_2$ is actively being reduced; that is,

only under conditions which permit the existence of bound $N_2$ reduction intermediates (268, 311, 339, 366, 690). Very low levels of $N_2$ will suffice (268); failure to recognize this may be responsible for the failure in one report (367) to demonstrate a $N_2$ requirement. The nitrogenase model described in Ref. 546 provides a striking analogy to the enzyme in that only the isolated diazene and hydrazine intermediates permit the exchange to occur, and participation by the N-bound H atoms is clearly indicated.

*2. Azide*

Azide undergoes a 2-electron reduction in what must be considered a relatively facile reaction with nitrogenase, as shown by a low $K_m$ (0.2-1.0 m*M*) and high electron allocation coefficient (about 0.70). Since various functional groups of proteins are known to be attacked by azide, its utility for evaluating nitrogenase function may be limited; however, concentrations of up to 10 m*M* did not adversely affect the reductant-dependent ATP utilization activity of nitrogenase (289). Azide ion is the predominant species at reaction pH, but whether this or $HN_3$ is the active species has not been established. Dinitrogen and ammonia are formed in approximately equimolar amounts, indicating that the enzyme is unable to retain product $N_2$ at the active site for further reduction. Azide reduction has been demonstrated with extracts of *Clostridium* (289, 599), *Azotobacter* (289, 369, 599, 621), *Bacillus* (144, 369), *Klebsiella* (144, 369, 540), *Rhodospirillum* (507) and soybean nodules (386), but not with intact organisms.

*3. $N_2O$*

Nitrous oxide exhibits many of the substrate characteristics of azide, including relatively low $K_m$ (1 m*M*) and high electron allocation coefficient (about 0.70) (287). In analogy to the reduction of azide to $N_2$ and $NH_3$, $N_2O$ is reduced to $N_2$ and presumably $H_2O$ with very little $NH_3$ detected as a product. As with azide reduction, $N_2$ produced from $N_2O$ is not favored for further reduction. The relative absence of $NH_3$, as well as the absence of inhibitory NO, indicates that bond cleavage is restricted to the NO bond, and presumably the substrate species is the relatively unstable resonance form $^{2-}N\text{-}N^{+}\equiv O^{+}$. Conclusions based on the absence of NO are equivocal, however, because of the ability of dithionite (present in the reaction mixtures) to reduce NO to $N_2O$ non-enzymically (287), and thus the possibility exists of some NN bond cleavage, presumably from the substrate species $N\equiv N^{+}\text{-}O^{-}$. On the other hand, the electron balance observed in $N_2O$ reduction indicates that this sequence does not occur significantly, if at all. The reduction of $N_2O$ has been demonstrated with nitrogenase preparations from *Azotobacter* and *Clostridium* (287), and was early detected in intact nodules and *Azotobacter* cells (p. 71), though the involvement of nitrogenase was at that time considered questionable.

*4. Nitriles*

The nitriles can be considered in three subclasses, grouped according to the characteristics of their reactivity with nitrogenase. These are cyanide, alkyl cyanides and alkenyl cyanides. The characteristic reduction for all nitriles involves reductive cleavage of the $-C\equiv N$ bond to yield ammonia and the hydrocarbon residues. Variations of this characteristic behavior have already been mentioned in the case of cyanide; the further reduction of unsaturated nitriles is described

below. The increase in the electron allocation coefficient of both alkyl and alkenyl cyanides attributable to $D_2O$, mentioned earlier (p. 121(, appears to be uniquely chracteristic of the reduction of these nitriles.

a) Cyanide

As previously pointed out, cyanide is a particularly interesting substrate because of its ability to function as a multi-class substrate. Like azide, its utility in nitrogenase analysis is complicated by its tendency to react nonspecifically; however, reductant-dependent ATP-utilizing activity is not altered at concentrations of the order of 5-10 m*M*. It is an effective substrate in terms of low $K_m$ (0.4-1.0 m*M*), but its electron allocation coefficient of about 0.35 is lower than those of the foregoing substrates; however, this value may be understated since electron balance in cyanide reductions indicates the probability of undetected products (289). At reaction pH, both HCN and $CN^-$ are present, although $CN^-$ is by far the major component. The multi-class behavior of cyanide suggests that both HCN and $CN^-$ may be substrates, with reductions proceeding along different lines for each to produce the variety of observed products (289, 372). The 6-electron reduction of cyanide to methane and ammonia is clearly the most favored reaction, accounting for about 90% of detectable electron consumption; the formation of another product tentatively identified as methylamine and involving addition of 4 electrons accounts for most of the remainder with only traces of $C_2H_6$ and $C_2H_4$ formed. The two latter products indicate class 5, isonitrile, behavior of cyanide, and these may be formed by the insertion reaction proposed for isonitrile reductions (p. 130). Cyanide reduction has been demonstrated with extracts of virtually all $N_2$-fixing agents used in nitrogenase analysis, but has not been reported for intact organisms; in view of its widespread interaction with metallo-proteins, major activity would not be expected in living organisms.

b) Alkyl Cyanides

The linear alkyl cyanides up to *n*-butyronitrile can function as nitrogenase substrates, but the branched compound, isobutyronitrile, cannot (286). The alkyl cyanides are considerably less effective substrates than the parent compound, as shown by the high $K_m$ values (>500 m*M*) and low electron allocation coefficients (<0.01). With respect to the latter parameter, however, it should be pointed out that because of the high $K_m$ values, electron allocation could be evaluated only at less than saturating substrate levels. Ammonia and the corresponding alkane are consistently produced in reduction of these substrates, with no apparent deviation. Reduction of the alkyl cyanides has been demonstrated with extracts of *Clostridium* and *A. vinelandii* (286).

c) Alkenyl Cyanides

The alkenyl cyanides are much more effective substrates than their alkyl cyanide analogs, possibly because the olefinic group can coordinate or react with nitrogenase to give a more effective enzyme-substrate complex. Alternatively, the double bond may enhance the reactivity of the nitrile group.

Acrylonitrile. Of the five unsaturated nitriles shown to be reduced by nitrogenase, acryolonitrile is by far the most effective substrate (241). The $K_m$ of acrylonitrile is much lower (10-50 m*M*) than those of other alkenyl cyanides, although it is higher than that normally encountered for a natural substrate of an enzyme. Its electron allocation coefficient of about 0.20 is relatively high. The variations in electron allocation coefficient with $D_2O$, ionic strength, pH and (at inhibitory acrylonitrile concentrations) with time have been mentioned (p. 122). Enhancement of the electron allocation coefficient by $D_2O$ was suggested to be due to the relative activation energies of electron transfer to $H_3O^+$, $D_3O^+$ or substrate, with $H^*_{D_3O^+} > H^*_{H_3O^+}$ In the case of acrylonitrile, and by analogy other alkyl and alkenyl nitriles, it was suggested that $H^*_{acrylonitrile}$ is similar to $H^*_{D_3O^+}$ but $>H^*_{H_3O^+}$, so that when $H_2O$ is replaced by $D_2O$, the difference between $H^*_{D_3O^+}$ and $H^*_{H_3O^+}$ is a major determinant in the electron allocation to acrylonitrile. The absence of a $D_2O$ enhancement in non-nitrile reductions is attributed to $H^*_{substrate}$ being dissimilar to $H^*_{D_3O^+}$ and $H^*_{H_3O^+}$ so that replacement of $H_2O$ by $D_2O$ would not significantly alter the relative allocation of electrons to substrate *vs.* $H_3O^+(D_3O^+)$. Effects of pH and ionic strength on electron allocation coefficient indicate that the site for $H_3O^+$ reduction is less sensitive and presumably less complex than the site for nitrile reduction.

The reduction products of acrylonitrile are ammonia, propylene and propane. The propane is not formed from product propylene, since the propylene:propane ratio did not increase with time. It thus appears that in the normal course of acrylonitrile reduction a portion of the bound substrate undergoes complete reduction before release. Though the acrylic double bond is not reduced when propylene is the product, it is shifted so that:

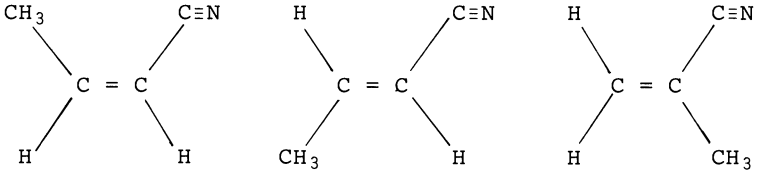
$$CH_2{=}CH{-}C{\equiv}N \xrightarrow{6D} CH_2D{-}CH{=}CD_2 + ND_3$$

The electron transfers involved in the reductions are $6e^-$ for the production of propylene + $NH_3$ and $8e^-$ for propane + $NH_3$. No evidence was found for 2 or 4 electron addition products in acrylonitrile reduction. The ratio of products, propylene:propane, was constant for given reaction conditions, but increased with $[H_3{}^+O]$ and ionic strength; under normal reaction conditions a ratio of about 6 was observed with *Azotobacter* preparations. The characteristics of the acrylonitrile reduction reaction are consistent with two suggested mechanisms for propane formation. One involves an allyl-enzyme intermediate which could undergo hydrolysis to yield propylene or, alternatively, could undergo further reduction to yield propane; the other involves an enzyme bound propionitrile intermediate which would be reductively cleaved to yield propane + $NH_3$ (241).

Though acrylonitrile is known to react with sulfhydryl groups, no inhibition was indicated at concentrations up to 10 m*M*. The nature of its reduction by nitrogenase clearly has significance for the interpretation of nitrogenase mechanism. Acrylonitrile reduction is also of considerable interest in that it has been demonstrated not only in *Azotobacter* extracts, but in intact $N_2$-grown *Azotobacter* cells as well, and furthermore the propylene:propane ratio is observed to be essentially the same *in vitro* as *in vivo*. This latter similarity is all the more remarkable in view of the sensitivity of this ratio to numerous milieu variations, and it stands as perhaps the best available evi-

dence that the nitrogenase reaction studied *in vitro* validly reflects the reaction *in vivo*. Acrylonitrile reduction also has been demonstrated with intact cells of *Clostridium* (241).

Methyl substituted acrylonitrile analogs. The monomethyl derivatives of acrylonitrile comprise the substrate series:



*cis*-crotonitrile *trans*-crotonitrile methacrylonitrile

In an absolute sense these are very poor substrates with characteristically high $K_m$ values and low electron allocation coefficients, and they clearly indicate the deleterious effect of substrate size on reaction rate. Within the series, the relative electron allocation coefficients show *cis*-crotonitrile to be a substantially more effective substrate than the *trans*-isomer, which is more effective than methacrylonitrile (276, 286). The relative effectiveness of these analogs as substrates has been used as a basis for evaluating the topography of the enzyme binding site (p. 149). Though the comparison is based on allocation of electrons under what were non-saturating conditions, it is likely that the indicated sequence is qualitatively, if not quantitatively, valid.

The reduction products of these substrates are $NH_3$ plus the carbon chain as either the alkene or alkane, though only the alkene product of methacrylonitrile is produced. The shifted double bond, which is characteristic of the propylene produced in acrylonitrile reduction, is also observed in the butene produced from *cis*- and *trans*- crotonitrile. 1-Butene and a possible trace of *n*-butane are produced, the latter constituting a possible exception to the general observation that only double bonds which are in conjugation with a triple bond are reducible by nitrogenase (286).

*5. Isonitriles*

The isonitriles are highly reactive substrates (286, 372), possibly because the high electron availability associated with the terminal C may facilitate enzyme-substrate complexation. Three linear isonitriles have been tested (286, 292, 366, 368, 372), and these show the same trend of activity as was seen for nitriles, with the lowest molecular weight species, methyl isocyanide, the most effective substrate and the unsaturated species, vinyl isocyanide, more reactive than the corresponding saturated species (368). Methyl- and ethylisonitrile are about 100 times more reactive than their nitrile isomers, aceto- and propionitrile. In support of complexation *via* electrons of the terminal C, a limited evaluation of vinyl isocyanide indicates it is more equivalent in reactivity to its nitrile isomer, acrylonitrile. Vinyl isocyanide has a substantially lower $K_m$ than acrylonitrile (about 0.8 m*M* *vs.* 10-50 m*M*), but its electron allocation coefficient is about the same.

The products obtained in isonitrile reductions indicate the operation of a reductive mechanism not detectable with other substrates, with

the exception of HCN. In these reductions 1- to 3-carbon alkanes and alkenes are generated from the isonitrilic carbons; the reduction products represent transfers of 6, 8, 10, 12 and 14 electrons. In addition to the isonitrilic C products, the amine residue of the isonitrile substrate is produced. The existence of other, unidentified, products must be considered a possibility (as in cyanide reduction) in the absence of a complete electron balance. The mixture of hydrocarbon products has been explained in terms of an alternating insertion-reduction mechanism (276, 286, 292) which proceeds through the following steps:

1. complexation of nitrogenase with the isonitrilic C of an initial isonitrile molecule to give $RN{\equiv}C^1$-enzyme;

2. six-electron reductive cleavage of the triple bond to yield free amine and methyl-nitrogenase, $H_3C^1$-E,

3. which could be hydrolyzed by proton attack to yield methane, or which could alternatively

4. insert a second isonitrile into the methyl-nitrogenase bond to create the intermediate

$$H_3C^1 - \underset{\underset{NR}{\|}}{C^2} - E$$ followed by

5. four-electron reductive cleavage of the double bond to yield

$H_3C^1 - C^2H_2 - E$ and then

6. either hydrolysis by proton attack to yield ethane, or hydride elimination to yield ethylene. Alternatively, the insertion of a third isonitrile could occur into the methylene-E bond, followed by four-electron reductive cleavage of the double bond to yield propyl-nitrogenase, which could yield propane or propylene by the preceding mechanism.

This sequence is supported by kinetic analyses which show that the rates of formation of the $C_2$ and $C_3$ products relate to the concentrations of isonitrile, $CH_4$ and $C_2$ products as

$$dC_2 \text{ products}/dt = K\,[\text{isonitrile}]\,dCH_4/dt$$
$$dC_3 \text{ products}/dt = K\,[\text{isonitrile}]\,dC_2 \text{ products}/dt.$$

Furthermore, the rate constants increase with a decrease in proton concentration (by increasing pH or replacing $H_2O$ with $D_2O$) and are altered by the action of competitive inserting agents such as CO and $CH_2O$ (276, 292). Mass spectral analysis of the methane formed in the presence of $D_2O$ shows only $CD_4$, thus implicating exclusively the isonitrilic carbon (372); comparable analyses of the $C_2$ products also indicated only deuterated ethane and ethylene (366).

Variations in the ratio of alkane:alkene products with isonitrile concentration, inhibitor (NO or CO) concentration, and with Mo-Fe:Fe protein ratio were interpreted as evidence for the operation of an undefined alternate reduction mechanism (366, 368, 369), and it appears probable that alternate minor pathways may exist for isonitrile reduction. The alkane:alkene product ratio also has been shown to vary according to the source of the nitrogenase tested and heterologous recombination experiments indicate that the ratio is specified by the Mo-Fe protein (369).

Isonitrile reductions have been demonstrated with extracts of *Azotobacter* (276, 290, 292, 366, 368, 369), *Clostridium* (272, 286, 292), *Rhodospirillum* (507), *Klebsiella* (369), *Bacillus* (369) and *Mycobacterium* (58); in addition methane production from methyl isonitrile has been observed with intact cells of *Azotobacter*, *Derxia*, *Clostridium* and *Desulfovibrio*, and also with intact clover nodules (366).

*6. Alkynes*

The ability of nitrogenase to reduce $C_2H_2$ has had a widespread impact in $N_2$ fixation research, both in the laboratory and in the field (274). The characteristics of the reaction and of product analysis combine to make $C_2H_2$ reduction the basis for a nitrogenase assay that is close to ideal (285; 10). Under normal assay conditions $C_2H_2$ is the most effective alternate reducible substrate known; its $K_m$ (0.1-0.3 m*M* or 0.004-0.010 atm) is comparable to that of $N_2$, but its electron allocation coefficient is close to a maximum 1.00 (242, 276, 301), by far superior to any other reducible substrate, including even $N_2$. It is thus the only substrate whose reduction is not accompanied by significant evolution of $H_2$. High levels of Fe protein, ATP or dithionite were observed to increase the $K_m$ (53, 104); additionally, pH influenced $K_m$, but no consistent pattern was evident (53).

Other alkynes which were reduced by nitrogenase are propyne and 1-butyne, but not 2-butyne (286). Allene also is reduced and is included among the alkynes on the grounds that it is a "potential" alkyne *via* isomerization to propyne, $H_2C{=}C{=}CH_2 \rightleftharpoons H_3C{-}C{\equiv}CH$; however, nitrogenase does not catalyze the isomerization to give free propyne, nor does isomerization of the enzyme-bound species appear to precede reduction since reduction in $D_2O$ leads to $CH_2D{-}CD{=}CH_2$, not $CH_3{-}CD{=}CHD$ (106). The $K_m$ and electron allocation coefficient for allene were evaluated at 0.43 atm and 0.72, resepctively.

Reduction of the alkynes proceeds exclusively *via* two-electron additions to yield the corresponding alkenes, but no alkanes. Reduction of acetylene in $D_2O$ yields *cis*-dideutero-ethylene with only traces of the *trans*-isomer, showing that the reaction is highly stereospecific and that the acetylenic H atoms are retained. Deuterated acetylene is reduced at the same rate as $C_2H_2$ and has the same $K_m$ (105), which suggests minimal participation of the H of acetylene in the reaction mechanism, though it is of course possible that these atoms are involved in a step which is not rate-limiting even when burdened by the isotope effect.

## Concluding Remarks on Substrate Reduction

No doubt other substrate members of the foregoing classes and possibly of new classes will be discovered. Examples which have been examined but for which no product was found include diazene generated *in situ*, hydrazine, methylamine, formaldehyde, methyliodide, cyanate, NO and CO (110, 272, 276). All known substrates cause a decrease in $H_2$ evolution, but at least at low concentrations do not affect ATP hydrolysis, and since the same behavior can be expected for undiscovered reducible substrates, candidates may be readily tested by comparing their effects on $H_2$ evolution and on ATP hydrolysis; effective substrates should inhibit the former but not the latter; possible products could then be sought from such substrates.

# Mechanism

The goal of much of the biochemical and biophysical research on $N_2$ fixation has been to define nitrogenase action at the molecular or active site level. The data in the preceding three chapters document this effort and tell a great deal about the composition and reactions of nitrogenase. Numerous mechanistic proposals have been made over the years, but it is still impossible to present a definitive mechanism for nitrogenase catalysis. The scheme of nitrogenase reactants and products shown earlier in Figure 4-5 incorporates the mechanistic implications of certain inhibitor studies and substrate reduction analyses, but it does not even begin to penetrate the subtleties of nitrogenase turnover. Nevertheless, there is good reason to expect that some of the mysteries of mechanism might soon be clarified. This optimism is prompted by the great backlog of descriptive data on nitrogenase that has accumulated as a base for further progress, by recent kinetic work and studies on specific enzyme-substrate interactions, and finally by the increasing attractiveness of various abiological $N_2$-fixing systems as possible models for the enzyme.

So far relevant experimentation has proceeded along two rather distinct lines, sufficiently unrelated that we have treated them separately in the present chapter. The first is essentially a reaction scheme approach and involves considerations such as reaction sequences, stoichiometry, and assignment of specific roles to the nitrogenase proteins; it has been pursued almost exclusively through biochemical and biophysical experimentation, and relevant information has accumulated slowly and somewhat randomly. Our interpretation of these data is embodied in a model reaction sequence that represents an initial effort to accommodate pertinent mechanistic data from numerous sources. The second major approach to nitrogenase mechanism is concerned with the characteristics of the reducible substrate binding site(s). The input here has been primarily biochemical and biophysical, but additionally inorganic chemistry has exerted an important influence on progress and thinking. This "active site" approach has been followed more intensively, and our present treatment of it constitutes an up-dating of earlier proposals.

## I. A Model for the Formation of Catalytically Active Species

The most pressing need in the pursuit of the over-all reaction mechanism of nitrogenase is a refinement of the basic reaction scheme of Figure 4-5. Until the exact nature and sequence of the interactions that occur among the various components of the nitrogenase reaction have been defined, little progress can be expected in understanding the mechanism of electron transfer and energy utilization. Although existing mechanistic data are limited it is possible at least to construct a model that is consistent with many of the reported find-

ings. These findings include the following features, which were described in Chapter 6:

1. Interaction between Fe protein and ATP-Mg;

2. Absence of interaction between Mo-Fe protein and ATP-Mg, except possibly in the presence of Fe protein and reductant;

3. Dilution effect, or decrease in specific activity with decrease in nitrogenase concentration, that is
   a) variable among preparations,
   b) induced by high ionic strength,
   c) induced by low ATP concentration;

4. Sigmoidal kinetics frequently observed for the saturation of Mo-Fe protein by Fe protein, and hyperbolic kinetics for the satuaration of Fe protein by Mo-Fe protein;

5. Absolute specificity for ATP;

6. ATP hydrolysis that
   a) requires both proteins and a divalent cation,
   b) is inhibited by ADP, but not AMP,
   c) is generally found to be optimal at an ATP:divalent cation ratio of 0.5 in the absence of an ATP-generating system,
   d) occurs at a low rate in the absence of reductant, but
   e) more rapidly when the nitrogenase proteins are reduced, and
   f) most rapidly during transfer of electrons from a reductant to $H_3O^+$ or other electron acceptor,
   g) is variable among preparations, but generally greater for recombined nitrogenases;

7. Electron transfer that is absolutely dependent on both proteins, a reductant, a divalent cation and the hydrolysis of ATP;

8. ATP:2e ratio that
   a) is variable among preparations,
   b) increases at high ratios of Mo-Fe protein to Fe protein because electron transfer is inhbited;

9. ATP saturation kinetics which are
   a) distinctly sigmoidal at low nitrogenase concentration, but
   b) nearly hyperbolic at high nitrogenase concentration;

10. Linearity of the relationship between $v^{-1}$ and $[ATP]^{-2}$ rather than $[ATP]^{-1}$;

11. Variation of apparent $K_m$ (ATP) with nitrogenase concentration;

12. Variations in $K_m$ values of reducible substrates with ATP concentration;

13. Influence of ATP concentration on electron allocation coefficient for $N_2$;

14. Biphasicity of Arrhenius plots;

15. Subunit structure of nitrogenase proteins;

16. Anomaly of three acidic proteins (ferredoxin, Mo-Fe protein and Fe protein) interacting;

17. Mössbauer and e.s.r. spectral responses of the Fe of the nitrogenase protein;

18. Large number of Fe atoms, but only one or two Mo atoms in nitrogenase;

19. Cross-reactivity among Mo-Fe proteins and Fe proteins from various diazotrophs that is less pronounced with proteins from anaerobic sources.

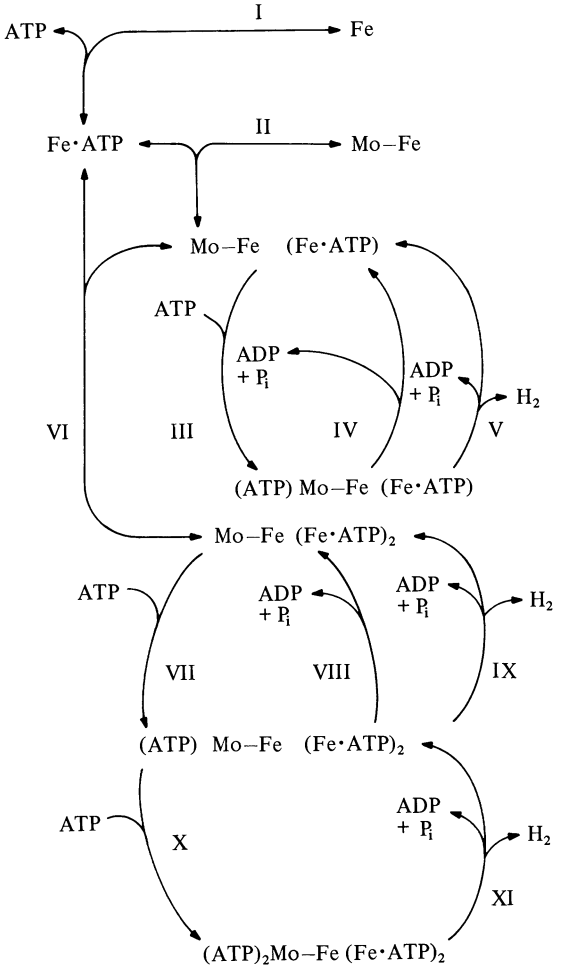



Fig. 7-1. A model for the formation of catalytically active nitrogenase species. Fe is Fe protein, Mo-Fe is Mo-Fe protein. See text for details

Based on these features of nitrogenase, we have constructed a model for nitrogenase action as shown in Figure 7-1. This model is proposed primarily as a working hypothesis for a reaction sequence, and its chief value may be as a test vehicle to promote further experimentation. The simplicity of the model reflects the current state of schematic sophistication in the field. The model is constructed to illustrate the least complex nitrogenase reaction, the reduction of $H_3O^+$ to form $H_2$, but it could be modified to show $N_2$ reduction, for example, by showing 2-electron transfer to Mo-Fe protein-bound $N_2$ (see next section) wherever reduction of $H_3O^+$ to $H_2$ is indicated. The model embodies many of the features just enumerated, and does not violate the rest. In all aspects it is consistent with reported data, though other interpretations are possible, and not all of the supportive data are firmly established.

The model contains elements of other schemes that have appeared in the literature. The initial interaction of Fe protein with ATP-Mg was proposed by MORTENSON et al. (495), who went on to suggest another type of ATP reaction involving both proteins; additionally, they inferred from e.p.r. spectral data that electron flow proceeds *via* dithionite → Fe protein → Mo-Fe protein. On the basis of kinetic data BERGERSEN and TURNER (53) advanced the concept that Fe protein functioned as an allosteric effector of Mo-Fe protein. The present scheme readily accommodates this interpretation, but does not necessarily endorse it. The model also incorporates features of the scheme described by SILVERSTEIN and BULEN (623) in which different active states of the enzyme are formed as the enzyme progressively becomes saturated with ATP.

The reactions of Figure 7-1 can be listed as follows, where Fe is Fe protein and Mo-Fe is Mo-Fe protein. For simplicity, the divalent cation has not been included.

I. $\text{Fe} + \text{ATP} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \text{Fe}\cdot\text{ATP}$ $\qquad k_1 > k_{-1}$

II. $\text{Fe}\cdot\text{ATP} + \text{Mo-Fe} \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} \text{Mo-Fe(Fe}\cdot\text{ATP)}$ $\qquad k_2 < k_{-2}$

III. $\text{Mo-Fe(Fe}\cdot\text{ATP)} + \text{ATP} \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} \text{(ATP)Mo-Fe(Fe}\cdot\text{ATP)}$ $\qquad k_3 < k_{-3}$

IV. $\text{(ATP)Mo-Fe(Fe}\cdot\text{ATP)} \underset{k_{-4}}{\overset{k_4}{\rightleftharpoons}} \text{Mo-Fe(Fe}\cdot\text{ATP)} + \text{ADP} + P_i$ $\qquad k_4 >> k_{-4}$

V. $\text{(ATP)Mo-Fe(Fe}\cdot\text{ATP)} + H_3O^+ \underset{k_{-5}}{\overset{k_5}{\rightleftharpoons}} \text{Mo-Fe(Fe}\cdot\text{ATP)} + \text{ADP} + P_i + H_2$ $\qquad k_5 >> k_{-5}$

VI. $\text{Mo-Fe(Fe}\cdot\text{ATP)} + \text{Fe}\cdot\text{ATP} \underset{k_{-6}}{\overset{k_6}{\rightleftharpoons}} \text{Mo-Fe(Fe}\cdot\text{ATP)}_2$ $\qquad k_6 < k_{-6}$

VII. $\text{Mo-Fe(Fe}\cdot\text{ATP)}_2 + \text{ATP} \underset{k_{-7}}{\overset{k_7}{\rightleftharpoons}} \text{(ATP)Mo-Fe(Fe}\cdot\text{ATP)}_2$ $\qquad k_7 > k_{-7}$

VIII. $\text{(ATP)Mo-Fe(Fe}\cdot\text{ATP)}_2 \underset{k_{-8}}{\overset{k_8}{\rightleftharpoons}} \text{Mo-Fe(Fe}\cdot\text{ATP)}_2 + \text{ADP} + P_i$ $\qquad k_8 >> k_{-8}$

IX. $\text{(ATP)Mo-Fe(Fe}\cdot\text{ATP)}_2 + H_3O^+ \underset{k_{-9}}{\overset{k_9}{\rightleftharpoons}} \text{Mo-Fe(Fe}\cdot\text{ATP)}_2 + \text{ADP} + P_i + H_2$ $\qquad k_9 >> k_{-9}$

X. $\text{(ATP)Mo-Fe(Fe}\cdot\text{ATP)}_2 + \text{ATP} \underset{k_{-10}}{\overset{k_{10}}{\rightleftharpoons}} \text{(ATP)}_2\text{Mo=Fe(Fe}\cdot\text{ATP)}_2$ $\qquad k_{10} > k_{-10}$

XI. $(ATP)_2Mo\text{-}Fe(Fe\cdot ATP)_2 + H_3O^+ \underset{k_{-11}}{\overset{k_{11}}{\rightleftharpoons}} (ATP)Mo\text{-}Fe(Fe\cdot ATP)_2 + ADP + P_i + H_2$ $\quad k_{11} >> k_{-11}$

The point(s) at which Mo-Fe protein and/or Fe protein get reduced is not critical, except that only reduced forms can proceed via reactions V, IX, and XI. The rate constants are as indicated; additionally, $K_4/K_5 > K_8/K_9$ (where $K_4 = k_4/k_{-4}$, etc.) and $k_3$ and $k_7$ are greater for the reduced proteins than for the oxidized (or less reduced) proteins.

The equilibria are such that under optimal conditions, $(ATP)_2Mo\text{-}Fe(Fe\cdot ATP)_2$ is the dominant species and reaction XI is limiting. Suboptimal levels of various reactants, or imbalance in the Mo-Fe protein: Fe protein ratio, will increase the concentrations of other species at the expense of $(ATP)_2Mo\text{-}Fe(Fe\cdot ATP)_2$, and reactions other than XI will become limiting. To some extent low concentrations of ATP will give rise to the same kinetic effects as low concentrations of Fe protein, because of reaction 1; however, the ATP effects may be more complex due to the additional involvement of ATP in reactions III, VII and X.

The complexes that are shown in model are based primarily on spectral and kinetic data. The reactivity pattern is derived mainly from kinetic results.

## A. Protein and Ligand Complexes of the Model

The Fe protein and Mo-Fe protein are depicted as co-existing independently in unsupplemented solutions. Electron micrographs support this contention, though the low protein concentration that must be used in the electron microscopic analyses is not representative of *in vitro* reaction mixtures and could lead to artifacts. The model does not preclude non-productive interactions among the proteins, but it does require the presence of substrates for the formation of catalytically active species, as described below. Some aspects of complex formation have been discussed earlier (p. 98).

### *1. Fe Protein·ATP·Mg Complex*

The existence of this complex is based on e.p.r. data (p. 92). A stoichiometry of 2 ATP·Mg per Fe protein dimer (55,000 daltons) was suggested, and the Fe protein of the model may be the dimeric form; electron micrographs show the Fe protein to exist primarily as the dimer or as dimer complexes, rather than as the monomer. However, in the absence of more definitive data, the Fe·ATP species of the model is not defined more rigidly than as an Fe protein·ATP·Mg complex. If it is in fact a complex of the dimer with two ATP·Mg, then reactionI should be followed by a reaction Ia showing $Fe\cdot ATP + ATP \rightleftharpoons Fe\cdot(ATP)_2$.

### *2. Mo-Fe Protein·Fe Protein Complexes*

There is no direct evidence to support the existence of these complexes, and they are presented primarily as a simple explanation for kinetic evidence, including titratration data and Hill plots (p. 108) that suggest functionality for 1 Fe protein:1 Mo-Fe protein complexes and for 2 Fe protein:1 Mo-Fe protein complexes.

## B. Reactivity of the Model

According to the model, the addition of ATP and $Mg^{2+}$ to a nitrogenase preparation will generate the Fe protein · ATP-Mg complex, but there will be little tendency for further reaction. The addition of reductant will promote reactions III, VIII and X (433). More significantly, because of the high rate constant, $k_{11}$, the reductant will tend to concentrate the nitrogenase proteins in the catalytically active Mo-Fe(Fe•ATP)$_2$ forms, primarily as the (ATP)$_2$Mo-Fe(Fe•ATP)$_2$ species. The model predicts that the fraction of nitrogenase proteins in the catalytically active form will be maximal at high concentrations of ATP and nitrogenase proteins, with the added stipulation that the Mo-Fe protein:Fe protein ratio is within an acceptable reaction range. To the extent that these conditions are realized, nitrogenase exhibits the classical, hyperbolic kinetics and reaction patterns characteristic of simple enzyme catalysis. Most of the unusual kinetic responses reported for nitrogenase can be explained in terms of deviation from these conditions which give rise to significant concentrations of other species.

### *1. Dilution Effect*

#### a) The Need for 2 Fe Proteins

In agreement with other authors, the model explains the dilution effect in terms of the need for two Fe proteins for optimal catalysis of electron transfer, but additionally the model proposes that the Fe·ATP complex, rather than free Fe protein, is the relevant species. Because the concentration of Fe•ATP is directly dependent on the concentration of Fe protein, a shortage of Fe protein will cause a shortage of Fe·ATP. Conversely, shortages of Fe•ATP can be overcome by supplementation with Fe protein, and the ability of supplemental Fe protein to overcome the dilution effect is well documented (p. 107). The involvement of two, rather than one, Fe proteins is supported by the sigmoidal relationship between activity and Fe protein concentration at constant Mo-Fe protein, by the value of 2 for Hill plots of log v/V-v *vs.* log Fe protein concentration, and by activity data that show an optimum molecular ratio of 2 Fe proteins per Mo-Fe protein (pp. 107-108). The dilution effect is thus a natural consequence of the dependency of reaction rate on the square of Fe protein concentration.

#### b) Effect of ATP Concentration

Many preparations of nitrogenase do not exhibit a dilution effect, presumably because Fe protein is not limiting in the reaction mixtures used. However, the effect can be produced in such preparations by decreasing the concentration of ATP (p. 109). This response is entirely consistent with the proposal that the Fe•ATP complex is a critical reactive species as described above and as illustrated in the model. Thus, low levels of ATP will give rise to a dilution effect by restricting the concentration of Fe•ATP. The effect of insufficient ATP in the presence of adequate Fe protein will be similar to the effect of insufficient Fe protein in the presence of adequate ATP. As with the Fe protein, the involvement of two ATP molecules is indicated, and kinetic evidence supports the concept that the nitrogenase reaction is bimolecular in ATP (p. 111).

c) ADP and Salt Effects

A response identical to the dilution effect is produced by ADP and by high salt concentration, as described earlier (p. 113). Though these effects are not incorporated in the model as it is presented, they are explicable within the context of Figure 7-1 as inhibitions of reactions which form the catalytic complexes. As a competitive inhibitor of ATP, ADP is implicated in reactions I, III, VII and X; the inhibition kinetics of high salt concentrations suggest interference with ATP, though the salt effects are not yet well defined and could involve inhibition of interactions between Mo-Fe protein and Fe protein. In view of the causes for the dilution effect proposed earlier, however, the inhibitions indicated for ADP and salt would readily produce this sort of effect.

*2. ATP Hydrolysis*

Reaction sequences leading to ATP hydrolysis occur in the model *via* reactions III and IV or V, VII and VIII or IX, and X and XI. Reactions IV and VIII constitute reductant-independent ATP hydrolysis, whereas reactions V, IX and XI reflect ATP hydrolysis that is associated with electron transfer. The ATP:2e ratio is then proportional to the ratio of reactions IV + V + VIII + IX + XI to reactions V + IX + XI. Since $(ATP)_2$Mo-Fe(Fe•ATP)$_2$ breaks down only *via* XI and since IX/VIII > V/IV, this ratio will be low when most of the nitrogenase proteins are in the Mo-Fe(Fe-ATP)$_2$ forms, and, conversely, it will be high when most are in the Mo-Fe(Fe•ATP) and dissociated forms.

The kinetic data of BUI and MORTENSON (88) indicate that a complex of one Fe protein per Mo-Fe protein is capable of catalyzing ATP hydrolysis, but not electron transfer. The present model proposes that such a complex can catalyze electron transfer as well as ATP hydrolysis, but does so less effectively than complexes containing 2 Fe proteins per Mo-Fe protein. LJONES and BURRIS (433) showed that excess Mo-Fe protein inhibited electron transfer giving rise to a higher ATP:2e ratio. According to the model this occurs because excess Mo-Fe protein would promote reaction II to such a degree that little Fe•ATP remains for reaction VI. The net result is an increase in the Mo-Fe(Fe•ATP) species at the expense of the Mo-Fe(Fe•ATP)$_2$ species, which in turn increases the ATP:2e ratio.

The frequent observation that the ATP:2e ratio is greater for recombined nitrogenase than for unfractionated preparations may reflect damage sustained during fractionation, which results in diminished ability to form the more efficient complexes. Additionally, the frequently observed higher rate of reductant-independent ATP hydrolysis catalyzed by recombined *vs.* unfractionated preparations could arise from the common practice of treating proteins with reductant during fractionation and purification. Enhancement of reductant-independent ATP hydrolysis by previous exposure of nitrogenase to reductant has been demonstrated (433).

*3. Nature of ATP Saturation Curves and Reciprocal Plots for $K_m$ (ATP) Evaluation*

As a result of the interdependency of ATP and Fe protein described previously, the decreased reaction rates associated with low ATP concentrations should be less apparent when the Fe protein concentration is relatively high. Experiments comparing the effects of high and low ATP at variable nitrogenase concentrations support this, but do not prove it

since unfractionated nitrogenase, rather than specifically Fe protein, was used (104). The data show that the ATP saturation curves become increasingly hyperbolic and less sigmoidal as the concentration of nitrogenase is increased, indicating that even at relatively low ATP concentrations, a substantial fraction of the nitrogenase in the high nitrogenase reactions is in the catalytically active form. Without the cushioning effect of a high nitrogenase concentration, a heightened sensitivity to low ATP levels is evident. This is seen in the pronounced sigmoidicity of ATP saturation curves obtained with low levels of enzyme; at low nitrogenase levels substantial amounts of ATP must be added before an appreciable level of catalytically active species can be formed. Anomalies in ATP saturation curves, such as the intermediary plateaus observed with lupine nitrogenase, suggest a complexity of ATP function that is not inconsistent with the present model.

It is evident from the ATP saturation curves that the degree of sigmoidicity parallels the concentration of ATP that is required for saturation. Thus, at low protein concentration, where the sigmoidicity is most pronounced, the apparent $K_m$ values are higher than at high protein concentrations (p. 110). Another consequence of the sigmoidicity is the linear relationship of $v^{-1}$ to $[ATP]^{-2}$ (p. 111).

*4. Reaction of ATP with the Mo-Fe Protein·Fe Protein Complexes*

The model proposes two different types of ATP interaction with the nitrogenase proteins. One, the reaction with Fe protein, is supported by e.p.r. data and is shown in the model to constitute an activation of Fe protein. The other, illustrated by reactions III, VII and X, depicts ATP reacting with multi-protein complexes in reactions which, unlike reaction I, lead to the hydrolysis of ATP. There is as yet no firm evidence that the Fe protein-ATP reaction is specifically an activating reaction and that a second type of ATP reaction is required to explain ATP hydrolysis. There is, however, some indirect support for two types of ATP reaction and for reaction between ATP and Mo-Fe protein complexes.

In support of two separate ATP reactions is the observation that nitrogenase reactions utilizing substrate levels of ATP proceed optimally when $Mg^{2+}$ is supplied at a level which assures the presence of both the ATP:$Mg^{2+}$ complex and also free ATP. This suggests that both species are required, and therefore that two different ATP reactions occur. Two separate ATP reactions are also suggested by the commonly observed slope of 2 for Hill plots of log v/V-v *vs.* log ATP concentration, as well as the sigmoidicity of ATP saturation curves and the linear dependency of ATP reciprocal plots on the reciprocal of the square of ATP concentration. These observations, however, could result from the need for 2 ATP molecules in the Fe protein activation reactions, as discussed earlier. Additional support for the reaction of ATP with an Fe protein·Mo-Fe protein complex capable of substrate reduction was obtained in e.p.r. experiments in which the addition of ATP·Mg altered the spectrum of Mo-Fe protein as well as Fe protein (495); this was interpreted in terms of an ATP reaction associated with substrate reduction, though the possibility cannot be ruled out that the altered spectra related to reductant-independent ATP hydrolysis reactions. A comparison of the $K_m$ (ATP) of reaction I with the $K_m$ (ATP) of the over-all nitrogenase reaction could be a key experiment in resolving the number of ATP reactions that occur.

## C. Allosteric Implications

The possibility that nitrogenase is an allosteric enzyme has been considered by a number of investigators, and it appears increasingly likely that this is the case. The following list of properties commonly associated with allosteric enzymes (480, 645) emphasizes this probability:

1. functionality at a key point in metabolism,
2. susceptibility to activator or inhibitor molecules called allosteric effectors, which are not necessarily metabolized in catalysis; two classes of allosteric effects are defined: "homotropic" interactions between identical ligands and "heterotrophic" effects between different ligands.
3. subunit structure,
4. quaternary structure that is influenced by enzyme concentration, ionic strength, temperature and presence of effector molecules,
5. cold sensitivity,
6. susceptibility to desensitization to allosteric control, and
7. sigmoidal, as opposed to hyperbolic, kinetics.

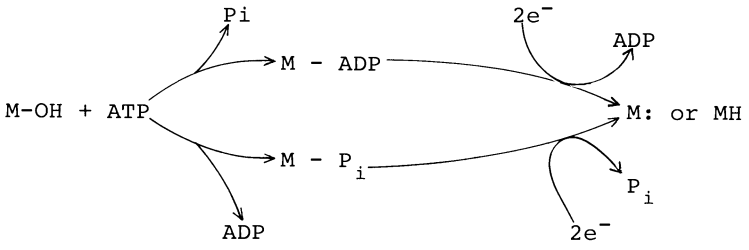
The nitrogenase model presented in this chapter can be interpreted in an allosteric context with little difficulty. This is particularly so with respect to ATP function, but can readily be extended to include an allosteric role for Fe protein as an effector of Mo-Fe protein, as has been suggested (53). The probability that proteins can function as effectors has been considered by MONOD et al. (480).

Certain aspects of nitrogenase behavior that are not reflected in the present model can be explained in terms of allosterism. Chief among these is the ability of various reaction components to influence the kinetic behavior of other reactants. Variations in the apparent $K_m$ of $N_2$ and $C_2H_2$ due to changes in concentration of ATP or reductant are examples (53, 104). Additionally, crude *Azotobacter* nitrogenase was reported to be sensitive to $NH_4^+$, but to lose this sensitivity following physical treatments that are known to cause desensitization to effectors in other systems (440); in our own work, however, we have not observed any specific sensitivity of crude *Azotobacter* nitrogenase to $NH_4^+$.

## D. The Mechanistic Enigmas of ATP Hydrolysis and Electron Transfer

The model makes no attempt to elaborate specific mechanisms in ATP function or electron transport. It is widely accepted that one function of ATP is to increase the reduction potential of a transient species in the electron transfer sequence, but no direct determination of potential has been made, only observations such as the failure of $H_2$ to inhibit electron transfer. One suggestion from our laboratory involves the utilization of ATP in creation of a highly unstable metallo-complex in which an ATP derivative is replaced by electrons to produce a hydride or its equivalent (277).

The sequence is based on the assumption that ADP or $P_i$ would be highly effective leaving groups which would provide a strong driving force for the reaction. The concept is supported by the reduction rates observed with a Pt complex (546) and by the ability of molybdo-thiol-




borohydride model of nitrogenase to evolve $H_2$ (602). Another role suggested for ATP involves the proton that is generated in ATP hydrolysis and the possibility that the reduction mechanism requires a very specific proton delivery that could be accomplished by this hydrolysis (342). The formation of an "active proton" in this way to facilitate protonation of $N_2$ would enhance subsequent electron acceptance by $N_2$ (128).

The electron transfer system within nitrogenase is no more well defined than the mechanism of ATP action. Dithionite produces changes in the electronic spectra of both proteins of nitrogenase. Inorganic models of the enzyme suggest that Fe is involved in electron transfer, and this concept is illustrated in a mechanism devised by SHILOV and LIKHTENSHTEIN (615) in which electrons reach Mo-bound $N_2$ from the electron donor *via* initial reduction of the Fe protein which in turn passes electrons to the Mo-Fe protein. The electrons travel through the Mo-Fe protein to $N_2$ by way of Fe-S transfer chains, and protons are visualized as originating from water and entering the reduction site by transmission through a coordinated base system. A major role for the Mo-Fe protein in electron transfer is supported experimentally by results with V-nitrogenase that indicate Mo to be intimately involved in electron allocation and probably functioning at the terminus of the electron transfer chain.

## II. Reducible Substrate Complexation and Reduction

### A. Locus of Active Site

Most evidence supports the Mo-Fe protein as the locus of the active site(s) for substrate reduction, though this point is not yet established unequivocally. In support of the Mo-Fe protein, for example, Hill plots of $N_2$ and $C_2H_2$ reduction showed that increases in the concentration of Fe protein (at constant Mo-Fe protein concentration) did not increase the number of active sites for $N_2$ or $C_2H_2$ reduction (53). Furthermore, it was shown that for nitrogenases composed of Mo-Fe protein from one organism and Fe protein from another, the isonitrile reduction products, ethylene and ethane, were produced in a ratio that was characteristic of the Mo-Fe protein (369), indicating that this protein was the site of isonitrile complexation and reduction. In other recombination studies, inhibition of $N_2$ reduction was observed when the isolated Mo-Fe protein was pre-incubated with CO, whereas, pre-incubation of the Fe protein with CO produced no inhibition (677).

The emphasis on metal participation in substrate binding has led to examination of abiological systems that bind and in some cases reduce $N_2$ or other nitrogenase substrates. The extent of metal-$N_2$

interactions was not fully appreciated until recently, but their study has now developed into an active research area in inorganic chemistry. Both biological and inorganic chemists have been quick to realize the potential significance of these systems as models for the nitrogenase reaction, and the possible biological implications of each inorganic development are being vigorously pursued. Conversely, the unique characteristics of nitrogenase provide guidance for inorganic models and serve as the criteria for evaluating the efficacy of these models.

The reactions of $N_2$ with inorganic agents are of several types, and these have been the subject of several recent reviews (128, 277, 615). The best known reaction is the Haber-Bosch reaction

$$N_2 + 3H_2 \underset{}{\overset{>400^{\circ},>700 \text{ atm } N_2}{\rightleftharpoons}} 2NH_3.$$

Because of the high temperature and pressure and the anyhdrous environment, this has least application to the enzymic system and will not be discussed further. The remaining types include nitriding, or Vol'pin and Shur systems, transition metal-$N_2$ complexes, and homogeneous protonic catalyses.

## B. Role of Metals and Implications of Abiological Systems

The idea that the metals of nitrogenase participate in the reducible substrate complexation is strongly ingrained in the nitrogenase literature. Once taken mainly on faith, this concept is now well supported by considerable indirect evidence, though definitive proof is still lacking. Its acceptance is illustrated by a recent report in which the failure of CO to affect the Mo-Fe protein visible spectrum was taken as presumptive evidence that reducible substrates bind to Mo, the rationale being that that since Fe, but not Mo, contributes to the visible spectrum, then CO (and therefore reducible substrates) must not bind to Fe and, by elimination, they must bind to Mo (677). The most direct support for the participation of Mo in substrate binding lies in kinetic work with *Azotobacter* V-nitrogenase (p. 122). This work showed that acetylene, acrylonitrile and CO bound less effectively to V-nitrogenase than to Mo-nitrogenase; this difference was not observed for $N_2$-binding, but the possibility of $N_2$ complexing initially to Fe and then bridging to Mo was considered, and in fact has some support in the conclusions drawn from inorganic studies (below). Abiological systems are discussed in detail in Refs. 128, 276, 277 and 615.

### *1. Nitriding Systems*

The most effective nitriding systems for $N_2$ reduction employ Ti, but successful results have also been obtained with Mo, V, W, Fe, Mn and Co. These function in conjunction with very strong reductants, a feature which restricts them to aprotic media. Like nitrogenase, reduction can be accomplished at, or near, ambient temperature and pressure; additionally, nitrogenase substrates other than $N_2$ are reduced, and CO inhibits. In some cases, reactivity approaches a pseudocatalytic level. It is probable that nitride formation proceeds *via* an initial metal-$N_2$ complex, and the reduced products are then generated in the course of solvolysis in the presence of a strong reductant. Since little is known of the actual mechanisms involved, the relevancy of nitriding reactions to nitrogenase remains obscure. The inability of such systems to function in aqueous media suggests they may have little in common with the enzyme.

*2. $N_2$ Complexes in Aprotic Media*

Two types of $N_2$ complexes can be distinguished, both of which may have significance for nitrogenase. In one type one or two molecules of $N_2$ are complexed to a single metal (mono-hapto complexes) and in the other $N_2$ forms a bridge between two metals.

ALLEN and SENOFF (8) isolated the first $N_2$ complex, $[Ru(NH_3)_5]^{2+}$ in 1965 and since then an ever-increasing list of $N_2$ complexes has been developing. The stability of these complexes is highly dependent on specific co-ligands as well as on the oxidation state of the metal, which generally must be moderately low (-1 to +2). These factors, coupled with the high ionization potential of $N_2$ (15.6 eV), limit the number of $N_2$ complexes.

Metals which have been shown to complex $N_2$ are shown in Table 7-1. Many of the complexes have been characterized by X-ray analysis, and in all cases $N_2$ was found to complex in the end-on, or linear, configuration, M-N-N. The infrared stretching frequencies of the N-N bond

Table 7-1. Metals shown to form complexes with $N_2$ [a]

| | | | | | | |
|---|---|---|---|---|---|---|
| (Ti) | V [b] | Cr | Mn | (Fe) | (Co) | (Ni) |
| Zr | Nb | (Mo) | Tc | (Ru) | (Rh) | Pd |
| Hf | Ta | (W) | (Re) | (Os) | (Ir) | (Pt) |

[a] Refs. 277, 382; complexing metals are circled.
[b] Vanadium is functional in abiological $N_2$ reduction (614), but no $N_2$ complex of V has been isolated.

of complexed $N_2$ are all lower than the value of about 2300 $cm^{-1}$ for $N_2$, and this lowering is in fact the main diagnosis for $N_2$ complexation. In this and other respects, the $N_2$ complexes are similar to metal carbonyls, though CO complexes are much more common.

Dinitrogen is isoelectronic with CO, and a similar bonding scheme has been proposed for them (Fig. 7-2). Complexes are formed and stabilized by insertion of electrons from a $\delta$-orbital of the coordinating N atom into acceptor $\delta$-orbital of the metal and by back donation of d-electrons from the metal into $\pi$ anti-bonding orbitals of the N atom. Since the most effective complexation will occur when the interacting orbitals are of similar energy, this bonding scheme requires that the metal possess very low energy $\delta$-orbitals to match those of N and, additionally, high energy, filled d-orbitals. Though the conditions are by no means mutually exclusive, they are in general not easily met and require specific co-ligands and metal oxidation state.

The Co complex, $CoH(N_2)(PPh_3)_3$, is the most thoroughly examined stable mono-hapto complex, and its properties parallel certain characteristics of nitrogenase. For example, $N_2$ is replaceable in the complex by $H_2$,

$$CoH(N_2)(PPh_3)_3 + H_2 \rightleftarrows CoH_3(PPh_3)_3 + N_2.$$

and to a lesser degree by ammonia,

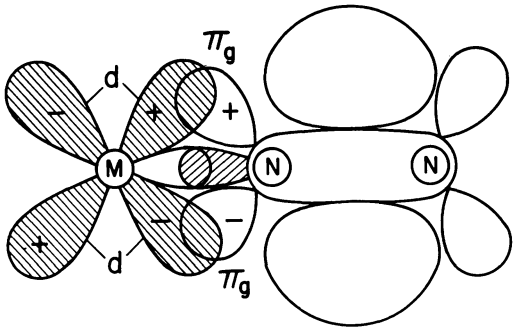



Fig. 7-2. Electronic representation of metal-$N_2$ bonding (from Ref. 277). The ligand-to-metal δ-bond arises from overlap of a filled δ-orbital ▨ of $N_2$ with a vacant δ-orbital of the metal. The metal-to-ligand π bond involves a filled metal d-orbital ▨ and a vacant πg molecular orbital of dinitrogen

$$CoH(N_2)(PPh_3)_3 + NH_3 \rightleftarrows CoH(NH_3)(PPh_3)_3 + N_2.$$

Additionally, ligand affinity characteristics resemble those of nitrogenase as shown in the following comparison which lists ligands in order of decreasing affinity (276).

Nitrogenase: $CO \sim NO >> N_2 \sim C_2H_2 \sim H_2 > N_3^-$, $N_2O$, RNC, RCN$>>NH_3$, $H_2O$, $C_2H_4$

$CoH(N_2)(PPh_3)_3$: $CO >> N_2 > H_2 > C_2H_4$, $NH_3$.

Reduction of ligating $N_2$ to ligating $N_2H_2$ has been demonstrated with Mo and W complexes containing two molecules of $N_2$ per metal (127), but at present the Co complex is unique among the mono-hapto $N_2$ complexes as the only one for which reduction of $N_2$ to $NH_3$ has been demonstrated (698). This requires strong reductants of the type used in nitride reductions, and reduction may in fact proceed *via* nitriding of released $N_2$. The reaction cannot be considered catalytic, but the fact that it can be accomplished helps remove one of the more serious objections to accepting the mono-hapto complexes as nitrogenase models.

As work on inorganic $N_2$ complexes developed, it was soon discovered that $N_2$ was capable of complexation with certain systems as a bridge between two metals. The initial bridged complex, $[(NH_3)_5Ru(N_2)Ru(NH_3)_5]^{+4}$, was isolated by HARRISON and TAUBE in 1968 (294), and since that time complexes have been identified in which $N_2$ bridges Re-Os, Mo-Mo, Ni-Ni, Re-Ti, Re-Ta, Re-Cr, Re-Co, Re-Mo, Re-Pt, Re-Al and Fe-Fe (602). In all of these a M-N-N-M' configuration is indicated.

The $N_2$ infrared stretching frequencies of these complexes vary from just under that of free $N_2$ to such exceptionally low values of 1680 $cm^{-1}$ for a $Re(N_2)Mo$ complex (125) and 1760 $cm^{-1}$ for a $Fe(N_2)Fe$ complex of the probable structure

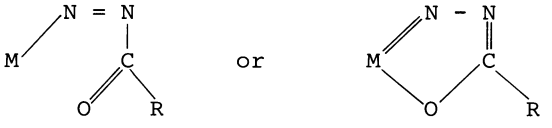
$$(Ph_3P)_2\,\underset{\displaystyle H}{\overset{\displaystyle R}{Fe}} - N \equiv N - \overset{\displaystyle R}{Fe}(Ph_3P)_2.$$

The di-iron complex is unique as the only $N_2$ bridging complex in which the $N_2$ has been reduced (to $N_2H_4$) (74); however, as in the reduction of the mono-hapto Co complex of $N_2$, strong reductants are required, and the reaction may proceed *via* initial release of the bridging $N_2$ and subsequent nitride formation and reduction.

Dinitrogen bridging complexes are not restricted to dimetal species, and in fact the first bridging species to figure in nitrogenase model work is the aryl-diazene-Pt complex (546)

$$[ArN{=}N\text{-}PtCl(PEt_3)_2]^+$$
$$\quad\quad\quad

Two additional types of mono-metal *bis*-$N_2$ bridging complexes have been described, both involving reactions of mono-hapto $N_2$ complexes. In the one case (126) compounds of the type



are formed by reaction with organic acyl chlorides; in the other (124) the adduct is $PF_5$ which forms a complex of exceptionally low infrared stretching frequency (1640 $cm^{-1}$) with *trans*-$[ReCl(N_2)(PMe_2Ph_4)]$.

*3. Homogeneous Protonic Catalysis*

The models in this category appear to reflect nitrogenase activity most closely. Two well-documented examples have been reported. In one, simple Mo or V compounds reacted with reductants comparable to dithionite at moderate temperature and pressure to produce $N_2H_4$ and also $NH_3$ (614). Stepwise electron transfer was indicated, with the initial 4-$e^-$ reduction preceding the secondary reduction of $N_2H_4$ to $NH_3$. The functional oxidation levels of the metals were presumed to be $Mo^{+3}$ and $V^{+2}$, both of which represent $d_3$ electron states. Reduction was genuinely catalytic, and the suggested mechanism involves an $N_2$ bridge between two Mo (or V) atoms. Specific similarities to the reactivity pattern of nitrogenase include stimulation by $Mg^{2+}$, evolution of $H_2$, reduction of $C_2H_2$ to $C_2H_4$ and competitive inhibition by CO. Based on the characteristics of these systems, Mo was assigned a central role in the enzymic complexation and reduction of $N_2$, and Fe was suggested to function primarily in electron transfer.

The most extensively examined model is the Mo-thiol system of SCHRAUZER and co-workers (601-607). This system has been tested against most aspects of nitrogenase activity and an astonishing similarity is evident, though the low rate of its reactivity with $N_2$ remains a major drawback. Molybdate plus a thiol such as cysteine or 1-thioglycerol provides an effective catalyst, though alternatively, a complex such as $Na_2Mo_2O_4(Cys)_2 \cdot 5H_2O$ may be used. Dinitrogen is suggested to be complexed in side-on configuration, in contrast to the linear configuration demonstrated for all other complexes examined. Iron functions as a co-catalyst, presumably participating in electron transfer to Mo. A comparison of the substrate-reducing properties of the Mo-thiol system and those of nitrogenase is given in Table 7-2.

## C. Active Site Model and Reduction Sequence

At present our picture of the active site(s) for substrate complexation and reduction must be built up from relevant information provided by inhibition studies, alternate substrate reduction characteristics, metal substitutions, HD exchange, synthetic $N_2$ complexes and model homogeneous protonic catalyses. The pertinent inhibition findings are that 1. the reducible substrates inhibit each other (p. 125), 2. $H_2$ specifically inhibits $N_2$ reduction (p. 126) and 3. CO inhibits the reduction of all substrates (p. 123). The simplest conclusion from the mutual inhibition exhibited by the reducible substrates is that they all react at a common site. The fact that the inhibitions are not all competitive does not preclude this (p. 125), and it is suggested that a common site be accepted in the absence of additional data. The

specificity of $H_2$ inhibition for $N_2$ reduction suggests that there is a site common to, and unique to these two compounds, whereas the ability of CO to inhibit all reducible substrates suggests that there is a site common to all of them.

Table 7-2. Similarities of reactions of nitrogenase and molybdothiol model

| | | Nitrogenase | Molybdothiol [a] |
|---|---|---|---|
| Substrates | | $N_2$, $C_2H_2$, $N_3^-$, $N_2O$, RCN, RNC | |
| Products | | $NH_3$, $C_2H_4$, $N_2+NH_3$, $N_2+H_2O$ $RCH_3+NH_3$, $RNH_2+CH_4$, $C_2H_6$, $C_2H_4$, $C_3H_6$, $C_3H_8$ | |
| Electron additions | | 2, 4, 6, 8, 10, 12, 14 | |
| Requirements | | Nitrogenase<br>ATP<br>$M^{2+}$<br>Dithionite | Mo, Thiol<br>XTP stimulates<br>Borohydride or<br>Dithionite |
| Metal specificity | | Fe+Mo or V | Mo(V?) |
| Optimum pH | | 6-8 | 9+ |
| Activation energy (kcal) | | ~14 | 13-21 |
| Turnover no. (moles/min•mole Mo) | $C_2H_2$ | 200 | $5\times10^{-2}$ |
| | $N_2$ | 50 | $10^{-6}$ |
| | RNC | 25 | $5\times10^{-6}$ |
| $K_m$ (mM) | $C_2H_2$ | 0.4-1 | 0.3 |
| | $N_2$ | 0.1 | >>1 |
| | RNC | 0.2-1 | 8-500 |
| CO | | strong inhibitor | weak inhibitor |
| $H_2$ | | no effect | no effect |
| $H_2$ evolution | | ATP-dependent | XTP-stimulated |

[a] Refs. 601-607

These considerations are readily accommodated by an active site composed of two components, one responsive to all substrates and the other responsive only to $N_2$ and the inhibitor $H_2$. In view of the evidence implicating metals in nitrogenase function and in $N_2$ complexes, these two active site components in all probability are metals. Thus the active center takes on the character of a bi-metallo site consisting of one metal that is reactive with CO and all reducible substrates and another that is reactive only with $N_2$ and $H_2$ (though reactivity with CO is not precluded at this site). Reactivity of $N_2$ with both metals suggests that $N_2$ may bind first to one metal and then bridge to the other. The inorganic $N_2$ bridging complexes make this an especially attractive mechanistic feature. Particular reference is made to the low infrared stretching frequencies associated with ligated $N_2$ in some of these binuclear complexes, and additionally to the remarkable similarity between the HD exchange characteristics of the aryl-diazene and hydrazine Pt model and those of nitrogenase.

Fe is implicated as the initial and unique binding site for $N_2$ by comparisons of V-nitrogenase and Mo-nitrogenase activity which showed that the $K_m$ of $N_2$ was similar for both enzymes, while the $K_m$ values of $C_2H_2$ and acrylonitrile and the $K_i$ of CO differed with each of the enzymes (100). These same data also point to Mo as the binding site for the other substrates.

Based on stability considerations and bond distances, we have earlier proposed a Mo-Fe site bridged by S and functioning in $N_2$ reduction as illustrated in Figure 7-3. The reduction of substrates other than $N_2$

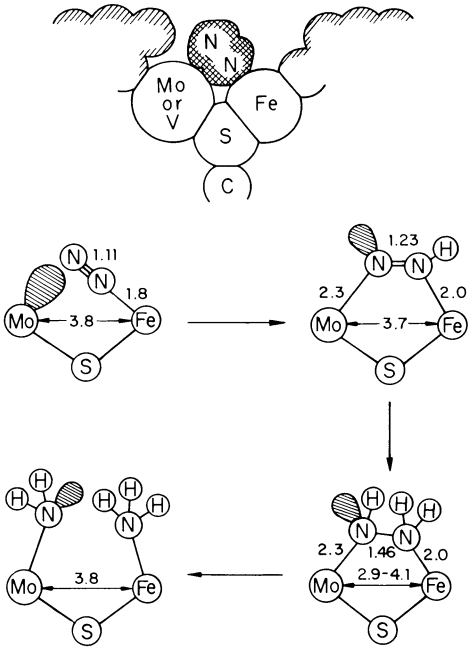



Fig. 7-3. Proposed site of reducible substrate complexation and reduction. The pocketed site (top) limits access to small molecules of appropriate shape. Stepwise reduction of $N_2$ is depicted below; distances are in angstroms (276)

is considered to occur at the Mo site. In this model $N_2$ binds initially to the Fe, then bridges to Mo. Reduction proceeds as electrons are added singly or in pairs. Addition in this stepwise manner is indicated by the fact that nitrogenase-catalyzed reductions are known to yield 2, 4, 6, 8, 10, 12 and 14 electron reduction products; additional support for stepwise reduction is provided by the aryl-diazene and hydrazine model mentioned previously, for which stable diazene and hydrazine intermediates have been isolated. The bridging S confers the stability of a 5-member ring on the system, but additionally it provides a flexibility which may help to accommodate an ever-increasing distance between the N atoms as reduction proceeds. This flexibility results from small changes in the Mo-S and Fe-S bond angles which allow a net increase in the Mo-S-Fe angle, as illustrated. Protonation of the bound $N_2$ and subsequent H-bonding to receptor site(s) on the enzyme could provide additional stabilization, perhaps sufficient to significantly diminish the activation energy associated with the addition of the first

pair of electrons to $N_2$; stabilization of hydroxymethyldiazenes in this way has been suggested (321).

Figure 7-3 shows the active site to be recessed into the enzyme, a localization that is prompted by the reduction patterns of the methyl-substituted acrylonitrile series (p. 130), and by isonitrile and alkyne analogs. The acrylonitrile analogs, *cis*-crotonitrile, *trans*-crotonitrile and methacrylonitrile, comprise a series in which the steric relationship between the bulky methyl group and the functional C≡N group varies. The wide range in the effectiveness of these compounds as nitrogenase substrates suggests that the active site is not equally available to the various isomers, and this selective accessibility is most simply explained in terms of a "pocketed" active site whose topography imposes steric restriction to entry.

# Cellular Accommodation of Nitrogenase

The nutritional flexibility of most organisms permits them to utilize a variety of different nitrogen sources, and adaptation to a particular source usually requires only relatively modest modification in cellular physiology and biochemistry. However, when the source nutrient is dinitrogen, the changes involved in adaptation can be particularly far reaching, with profound effects not only on the physiology and biochemistry of organism, but on its structure as well. Perhaps most striking in this context are the adaptations of symbiotic systems, which produce complex anatomical structures, such as root nodules, whose sole purpose is to utilize $N_2$. Even among the simplest $N_2$-fixing organisms, the bacterial diazotrophs, significant modifications may occur. The large amount of nitrogenase produced is itself noteworthy, with the enzyme comprising 5% or more of readily extractable cell protein. Auxillary proteins are produced and the ATP demands of $N_2$ fixation may require different or accelerated energy metabolism. Moreover, the sensitivity of nitrogenase to $O_2$ may necessitate specialized metabolism or structures to protect the enzyme from $O_2$. In this chapter observations dealing with the impact of $N_2$ fixation on cell structure and function will be summarized and discussed.

## I. Localization of Nitrogenase

The localization of nitrogenase has been examined at both the cellular and subcellular levels. With multicelled diazotrophs, studies have not yet proceeded beyond the cellular stage. Among these the enzyme occurs in the bacteriod cells of legume nodules and is indicated to occur in both heterocystous and vegetative cells of blue-green algae, with the distribution depending on $pO_2$ (p. 104). Attempts to define the subcellular locus of nitrogenase have been made mainly with *Azotobacter vinelandii*. To date these have not produced definitive answers.

In early localization studies with *Azotobacter*, dye-binding methods were used to identify centers of high reducing activity, and in this indirect manner structures possessing mitochondrial properties and designated as mesosomes were suggested as the loci of nitrogenase; however, these membranous structures could not be detected in intact cells and were found only in the cell fraction that sedimented at 187,000 × *g* (422, 733, 734). More recently, electron microscopy is being employed in localization studies. Comparisons of electron micrographs of *Azotobacter* grown on either $N_2$ or fixed nitrogen led to the conclusion that the $N_2$-grown cells developed a unique, extensive cytoplasmic membrane system on which nitrogenase was localized (530, 531). In subsequent electron micrographic analyses, however, others could find no significant differences among the intracellular membrane systems of cells grown on various nitrogen sources, including $N_2$ (649, 750). Work with *Anabaena* preparations suggested that the algal nitrogenase was not closely membrane-bound, but that in crude extracts it may be part of, or be adsorbed into, a large proteinaceous complex (301).

Numerous analyses of *Azotobacter* fractions indicate that both "particulate" and "soluble" nitrogenase preparations can be obtained, depending only on the method of cell rupture (530). The nitrogenase literature does not always define these solubility terms, but in all cases they relate only to how readily the activity can be sedimented, and it is not at all clear whether the particulate nitrogenase preparations are in fact intact nitrogenase loci with real physiological significance, or whether they are simply artifacts of preparation.

The problems of localizing nitrogenase within the cell appear to be primarily problems of methodology. While there may be some merit in pursuing the methods described above, it is important to note that a new approach is now possible. This involves the use of antibodies to the nitrogenase proteins, which are now available and can be conjugated with marker molecules (156, 649). Treatment of diazotroph cells or cell sections with such conjugates could be expected to form complexes with the Mo-Fe protein and Fe protein at their cellular loci and permit direct localization by electron microscopic examination (649).

## II. Electron-Donating Systems

Early cellular studies with diazotrophs provided strong circumstantial evidence that $N_2$ fixation was a reductive process (717), but the resulting data offered little insight into the metabolic source of electrons or the sequence of electron transfer to nitrogenase. With the advent of the $N_2$-fixing *Clostridium* extracts it was immediately apparent that pyruvate served as the ultimate electron donor for *Clostridium* nitrogenase. Fractionation of the *Clostridium* extracts led to the discovery of the non-heme iron protein, ferredoxin, and established this protein as the electron carrier which coupled such reductants as pyruvate, $H_2$ and formate to *Clostridium* nitrogenase (p. 66). The discovery of ferredoxin had ramifications far beyond the field of nitrogen fixation; as the first of many such proteins to be isolated and characterized, it was instrumental in initiating research in the group of proteins characterized by an Fe-S prosthetic group (see 744 and References therein). Each of the nitrogenase proteins is, of course, an iron-sulfur protein, and information on the Fe-S prosthetic group (Fig. 8-1) gained from ferredoxin studies could well be relevant to the nitrogenase proteins.

Application of the pyruvate methodology to extracts of other organisms appeared promising at first, then disappointing, as initial success with *Rhodospirillum* preparations proved difficult to reproduce. It is only recently, in fact, that support of nitrogenase by pyruvate has been shown for extracts of organisms other than *Clostridium*, with positive results now reported for *Bacillus*, *Azotobacter* and *Chloropseudomonas* extracts (see below).

Attempts to obtain apoferredoxin (ferredoxin minus its Fe) by growing *Clostridium* on limiting iron led to the discovery that under this nutritional restriction the bacteria produced, in place of ferredoxin, a flavoprotein with the functional characteristics of ferredoxin (387). This protein was called flavodoxin. As with ferredoxin, flavodoxins were subsequently detected in other organisms, including non-diazotrophs, and the extent of their occurrence and metabolic significance is still being explored. Flavodoxins from *Clostridium* (387, 388, 389) *Escherichia* (695), *Desulfovibrio* (197), *Rhodospirillum* (138), *Anacystis*

(76), *Peptostreptococcus* (456) and *Azotobacter* (38), the latter called azotoflavin, have been isolated and characterized and the literature on flavodoxin recently has been reviewed (744).

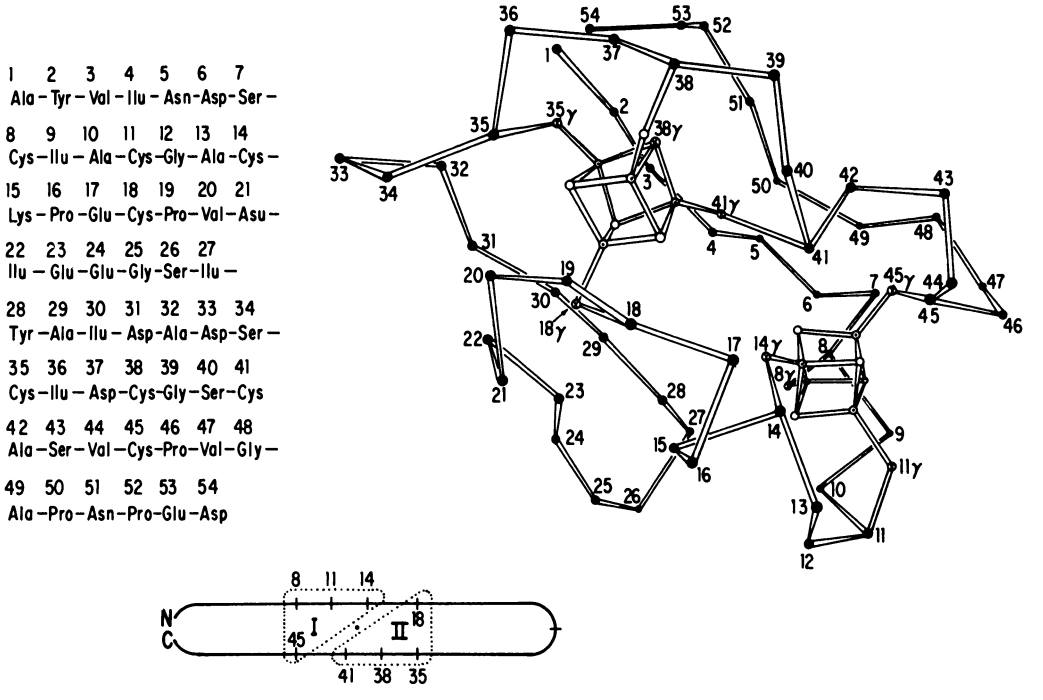



Fig. 8-1. "The structure of the 8Fe-8S ferredoxin from *Peptococcus aerogenes* represented by the positions of αcarbon, iron, inorganic sulfur and cysteinyl sulfur atoms deduced from the 2.8 Å three derivative isomorphous replacement electron density map. The pseudo twofold nature of the molecule inherent in the sequence and exhibited by the three-dimensional structure is schematically represented in the lower portion of the figure, which also illustrates the fact that each complex is formed from three cysteinyl sulfurs in one half of the chain and one from the other half." (Courtesy ADMAN, SECKER and JENSEN, Ref. 3)

The participation of ferredoxins and flavodoxins has been demonstrated with a sufficient variety of diazotrophs to suggest that they function with nitrogenases from all sources (Table 8-1). This supposition is supported also by the relative ease with which ferredoxins from various sources can function with different nitrogenases; interchangeability is particularly evident with *Clostridium* ferredoxin, which has been shown to couple with the nitrogenases of *Azotobacter, Klebsiella, Chromatium, Chloropseudomonas, Anabaena* and soybean bacteroids as well as *Clostridium*.

Although extracts from diazotrophs other than *Clostridium* failed to respond well to pyruvate, greater success was achieved by using *Clostridium* hydrogenase and ferredoxin to couple electrons from $H_2$ to nitrogenases from various sources. This method of electron transfer was successfully applied to extracts of *Azotobacter, Klebsiella, Chromatium* and soybean bacteroids, and its use with *Azotobacter* preparations led to the discovery of dithionite as a reductant for nitrogenase.

Table 8-1. Diazotrophs known to possess Ferredoxin or Flavodoxin (taken mainly from Ref. 744; also see Refs. 75, 638)

| Diazotroph | Ferredoxin | Flavodoxin |
|---|---|---|
| *Azotobacter vinelandii* | + | + |
| *Azotobacter chroococcum* | + | |
| *Clostridium pasteurianum* | + | + |
| Other *Clostridia* | + | |
| *Methanobacillus omelianskii* | + | |
| *Bacillus polymyxa* | + | |
| *Klebsiella pneumoniae* | | + |
| *Rhodospirillum rubrum* | + | + |
| *Chloropseudomonas ethylicum* | + | |
| *Chromatium* D | + | |
| *Chlorobium thiosulfatophilum* | + | |
| *Desulfovibrio gigas* | + | + |
| *Desulfovibrio vulgaris* | | + |
| *Desulfovibrio desulfuricans* | + | |
| *Rhizobium* (bacteroids) | + | |
| *Anabaena cylindrica* | + | |

Dithionite is now generally used for routine analyses of nitrogenases from all sources (p. 70). Studies with purified nitrogenases show that dithionite reduces the enzyme directly with no requirement for an electron-transferring agent. At present no other reductant is known which couples directly to nitrogenase and can be used in substrate amounts. The only other non-physiological agents which have been used successfully in nitrogenase reducing systems are methyl and benzyl viologen dyes, and these have been used only at catalytic levels. Methyl viologen can replace ferredoxin in *Clostridium* systems employing pyruvate or $H_2$ as the ultimate reductant (170), but it inhibits *Azotobacter* and *Chloropseudomonas* nitrogenases. Both methyl and benzyl viologen have been used to facilitate electron transfer to various nitrogenases from physiological metabolites, but their role in these systems is not well defined.

The ease with which nitrogenases from all sources can be reduced by dithionite has made this reagent extremely useful in nitrogenase analyses; on the other hand, this same facility of action disqualifies it for investigations of natural electron donor systems, since it so easily overrides these systems. To overcome this difficulty, photochemical reduction systems were developed (below), and these have proved increasingly useful. They consist generally of a chloroplast preparation lacking ferredoxin and heated to destroy the $O_2$-evolving function, plus an electron donor and a carrier dye; a typical mixture contains fragmented spinach chloroplasts, ascorbate and DCPIP (dichlorophenol-indophenol). Electron flow is readily regulated by light. Such systems have been applied to extracts of *Azotobacter*, soybean bacteroids, *Chromatium*, *Anabaena* and *Chloropseudomonas*, and have facilitated the identification of electron transfer components.

The electron-donating systems of individual diazotrophs are discussed below.

## A. Clostridium pasteurianum

The use of pyruvate with *Clostridium* extracts and the impact of the *Clostridium* pyruvate system on research in $N_2$ fixation have already

been discussed (p. 66). Other substrates that reduce ferredoxin are also effective, though the physiological significance of these is probably not great. Included are α-ketobutyrate (120), formate with formic dehydrogenase (489), NADH (170), and $H_2$ with hydrogenase (170, 486).

## B. Azotobacter vinelandii

Ultimate electron donors as well as electron-transferring agents have been reported for this diazotroph; however, activites observed are still low relative to the activity obtained with dithionite. *Azotobacter* nitrogenase activity is supported by NADPH, and a major physiological role for this cofactor as an electron donor for nitrogenase activity was suggested (39). The transfer of electrons from NADPH required four additional components: 1. a factor that was replaceable by spinach ferredoxin-NADP reductase, 2. a heat labile factor, 3. *Azotobacter* ferredoxin and 4. *Azotobacter* flavodoxin. The sequence of interaction among these components is undetermined. Substrate levels of NADPH were as effective as numerous NADPH generators (including glucose-6-phosphate, malate or isocitrate, plus the appropriate dehydrogenase), but NADH was ineffective. An early report (385) described the use of β-hydroxybutyrate, $NAD^+$ and viologen dye for electron transfer to nitrogenase, but no details or data were presented. More recently a role for pyruvate in anaerobic $N_2$ fixation by *Azotobacter* has been indicated, with pyruvate functioning as the ultimate source of both energy and reductant in a manner similar to that of *Clostridium* (267).

The use of chloroplast preparations to generate electrons photochemically for nitrogenase reduction facilitated the discovery of *Azotobacter* ferredoxin and flavodoxin (38, 743). The initial reports indicated that each of these carriers supported nitrogen fixation independently of the other when reduced by illuminated spinach chloroplasts. Subsequent data, cited above, showed both to be required for maximal activity with NADPH as reductant; it is not yet clear whether there is an absolute requirement for flavodoxin. Additional work with the illuminated chloroplast system has confirmed the earlier observations with *Azotobacter* flavodoxin and ferredoxin and extended the list of effective carriers in this system to include algal flavodoxin (phytoflavin) and *Anacystis* ferredoxin (426). *Azotobacter* flavodoxin has been further characterized (426), and the *Azotobacter* ferredoxin fraction may contain two different ferredoxins, both able to couple electrons to nitrogenase (741).

*Clostridium* ferredoxin couples effectively to *Azotobacter* nitrogenase and has been used to mediate electron transfer from $H_2$ (with *Clostridium* hydrogenase) (93), from spinach chloroplasts or chloroplast fragments (with ascorbate as the ultimate reductant) (39), and from NADPH (with spinach ferredoxin-$NADP^+$ reductase) (39).

## C. Azotobacter chroococcum

With NADH as electron donor either in substrate amounts or in catalytic amounts in conjunction with added carbon substrates and endogenous dehydrogenases, *A. chroococcum* extracts reduced low levels of $C_2H_2$ (736, 737). Effective substrates included glucose-6-phosphate, various sugars and organic acids; $H_2$ at 0.10 atm was ineffective. Acetylene reduction supported by NADH was observed with both the supernatant and pellet fractions obtained in the initial centrifugation of broken cell suspensions. The major difference between the activities of these two

fractions was the need for benzyl viologen specifically with the supernatant fraction; methyl viologen was about half as effective as benzyl viologen. Activities were uniformly low in NADH-supported reactions, ranging from <1 to 7% of the activities obtained with dithionite.

## D. Mycobacterium flavum

Results similar to those described for *A. chroococcum* were obtained with preparations of *M. flavum*. Supernatant fractions gave weak $C_2H_2$ and cyanide reducing activities with NADH as electron donor (59), and benzyl viologen was required. It could not be replaced by any of fourteen tested substitutes. Optimal level for benzyl viologen was 0.15 m*M* and higher concentrations were inhibitory. In the same investigation $C_2H_2$ reduction with NADH was also shown for "debris preparations" which were obtained by resuspending the material precipitated by the centrifugation of broken cells. As with the soluble preparations activities were very low, but unlike the soluble preparations, benzyl viologen was not required and was in fact inhibitory. In both systems lactate, pyruvate, glucose-6-phosphate and $H_2$ were able to replace NADH with various effectiveness. NADPH was not tested.

## E. Bacillus polymyxa

In analogy to *Clostridium* preparations, reduction of $N_2$ by extracts supplemented with pyruvate or formate has been demonstrated (232). Pyruvate was the better substrate and appeared to be functioning *via* the phosphoroclastic reaction, since its metabolism gave rise to ATP as well as reductant. Dihydrogen, NADH and NADPH failed to support activity. A ferredoxin has been isolated from extracts of $N_2$-fixing *Bacillus*, and a role for it in electron donation to nitrogenase is indicated by its ability to enhance $C_2H_2$-reducing activity when added back to ferredoxin-depleted preparations (613).

## F. Chromatium

Two different natural systems, both requiring ferredoxin, are effective with *Chromatium* nitrogenase. In one case electrons were generated photochemically using *Chromatium* chromatophores in combination with ascorbate and DCPIP (740). In the other $H_2$ was the ultimate electron donor and transfer to nitrogenase was *via Chromatium* hydrogenase and ferredoxin (721). *Chromatium* ferredoxin was effective in both systems, though *Clostridium* ferredoxin was more active in the $H_2$ system, the only place in which it was tested; *Chlorobium* ferredoxin could also be used, but spinach ferredoxin was inactive (739, 740). Activities were sufficiently high with both systems to measure nitrogenase by ammonia formation as well as by $C_2H_2$ reduction.

## G. Chloropseudomonas ethylicum

Two independent electron-donating systems have been demonstrated with crude extracts(218). In one pyruvate is the ultimate reductant, and in the other electrons are provided photochemically *via* the action of light on bacterio-chlorophyll. Both systems require ferredoxin. The pyruvate system is of questionable physiological significance, since cellular nitrogenase activity is ten times greater in light than in dark; however, *in vitro* pyruvate supported activity better than either

illuminated chloroplast particles or dithionite, though even the highest activty obtained (11 units $[C_2H_2]$) was low compared with *Azotobacter* or *Clostridium*. Inexplicably, added *Chloropseudomonas* ferredoxin stimulated activity supported by dithionite three- to fourfold; ferredoxins from *Chromatium* and *Clostridium* were less effective, while spinach ferredoxin had no effect and methyl viologen was inhibitory at 0.67 m*M*.

Photochemically generated reductant and an added ATP-generating system supported $C_2H_2$ reduction about 40% as well as did pyruvate. The ultimate electron donor was 2-mercaptoethanol, and chlorophyll-containing particles from either *Chloropseudomonas* or spinach were used.

## H. Anabaena cylindrica

In early experiments marginal $^{15}N_2$ reduction was obtained with extracts supported by pyruvate (137). Subsequent work implicated *Anabaena* ferredoxin in the transfer of electrons to nitrogenase from numerous reductants including chlorophyll, NADPH *via* ferredoxin-$NADP^+$ reductase, and $H_2$ *via Clostridium* hydrogenase (75, 301, 638). The pyruvate dehydrogenase of *Anabaena* appears to be $NADP^+$-dependent rather than ferredoxin-dependent (301, 638), and pyruvate functioned effectively as electron donor only when $NADP^+$ was supplied in addition to Fd (638). Other effective NADPH generating systems included isocitrate (638) and glucose-6-phosphate (76) with the appropriate dehydrogenases. Under normal physiological conditions it is likely that the only significant source of electrons for ferredoxin reduction and thus for nitrogenase, is reduced chlorophyll.

The ferredoxin obtained from *Anabaena* is described as a typical plant-type ferredoxin and thus is a one-electron transferring protein containing 2 Fe and 2 $S^{2-}$ (638). In contrast the ferredoxins associated with $N_2$ fixation in other diazotrophs are bacterial-type, containing 4 or more Fe and $S^{2-}$ and capable of one- or two-electron transfer. The possibility that a yet unidentified electron carrier functions with *Anabaena* nitrogenase has been considered, but there is no evidence for this (301). Other electron carriers which were effective in reducing *Anabaena* nitrogenase include spinach ferredoxin, *Anacystis* flavodoxin (76) and *Clostridium* ferredoxin (301). Slight activity was observed with benzyl, but not methyl viologen (301).

## I. Soybean Nodule Bacteroids

The ultimate reductant for soybean nodule bacteroids remains unknown, but a clearer picture of the electron-donating system is emerging. Maximal dinitrogen fixation appears to require fresh photosynthate (279, 280), an observation which also has been made with *Alnus* (707). Studies with field-grown soybeans indicate that photosynthate is in fact the major limiting factor in $N_2$ fixation in the field. Increases in net photosynthesis by $CO_2$-enrichment have dramatically enhanced $N_2$ fixation by 5-fold during the complete growth cycle (281, 282). The increased fixation is due to increased specific $N_2$-fixing activity of nodules and to increased nodule mass. The photosynthate may support $N_2$ fixation by providing energy, reductant and/or carbon skeletons for the incorporation of fixed nitrogen (Fig. 8-2).

Beta-hydroxybutyrate was once though to be a most likely candidate for the ultimate electron donor in nodules (385), but it is not longer considered for this role (46, 730), even though massive amounts are potentially available from the poly-β-hydroxybutyrate granules which

accumulate in the nodules. Succinate was reported to restore brief but intense nitrogenase activity to intact bacteroids (46), but has not yet been further investigated.

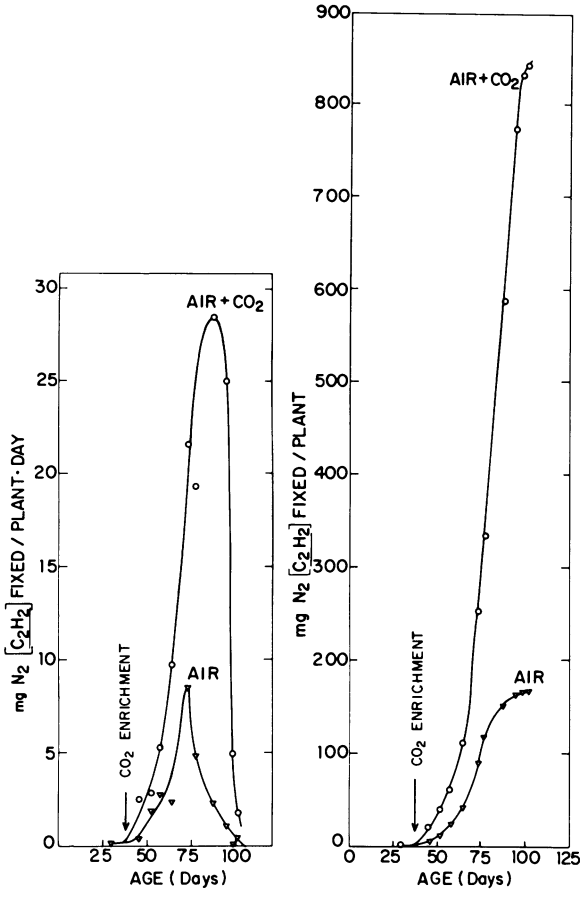



Fig. 8-2. Multifold enhancement of $N_2$ fixation by $CO_2$-enrichment of field-grown soybeans (Ref. 281, 282). $N_2$-fixing activities were measured weekly during the complete growth cycle by $C_2H_2$ reduction assay. $CO_2$-enriched plants were exposed to 800-1200 ppm $CO_2$

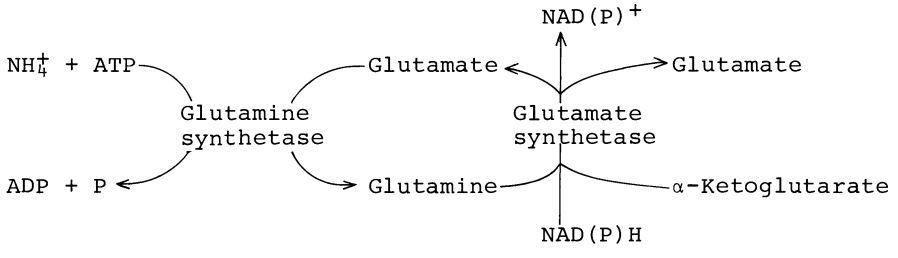
Early reports implicated NADH as an important link in supplying reductant to nitrogenase, and substrate levels of NADH were weakly effective (214, 385). More recently NADPH was indicated to be superior to NADH, and flavins have been implicated (730, 731). A ferredoxin similar to *Azotobacter* ferredoxin has been obtained from soybean nodule bacteroids (39, 396, 397), but how it may function in the foregoing sequence is not clear. Its activity has been demonstrated with photochemical electron-generating systems in which it was required to transfer electrons from illuminated spinach chlorophyll to bacteroid nitrogenase; the possible operation of other components in this electron transfer is not excluded. The bacteroid ferredoxin has not been characterized in detail yet, but it has been purified and shown to possess an uncommonly high sensitivity to $O_2$, losing 85% of its activity after 2 hrs exposure to 100% $O_2$ at $30^{o}$ (742).

The coupling of *Clostridium* ferredoxin to bacteroid nitrogenase has been demonstrated with a *Clostridium* hydrogenase-ferredoxin system that transfers electrons from $H_2$ to nitrogenase (397). Ferredoxin from *Azotobacter* (742), *Scenedesmus* (397) and *Clostridium* (397) have been successfully used in place of bacteroid ferredoxin in the photochemical reduction systems.

## III. Ammonia Assimilation

The early kinetic studies of $^{15}N_2$ incorporation that pointed to ammonium as the product of $N_2$ fixation additionally provided some insight into the manner of ammonia incorporation. Patterns of $^{15}N$ labeling showed major incorporation into glutamate, aspartate, "amide nitrogen" and alanine and indicated a central role for glutamic dehydrogenase and participation by glutamine synthetase, aspartate amino transferase and other transaminases, and possibly also a role for alanine dehydrogenase (108, 237, 373, 713). The limited work done with alder suggests ammonia incorporation by the carbamyl phosphate synthetase reaction (416); *Clostridium* extracts and the bacteroid fraction of soybean nodules also have vigorous carbamyl phosphate activity, but a significant role in the incorporation of fixed $N_2$ is not indicated (237, 722).

Recently NAGATANI, SHIMIZU and VALENTINE (509) presented evidence that the glutamine synthetase that occurs in diazotrophs is predominantly the poorly adenylated form, which is characterized by a low $K_m$ for ammonia. In this form the enzyme is ideally suitable for diazotrophs, since it assures efficient assimilation of fixed nitrogen and prevents ammonium accumulations which might inhibit nitrogenase biosynthesis. In conjunction with this glutamine synthetase the recently discovered enzyme, glutamate synthetase, was implicated in the following sequence:




Comparisons of the glutamic dehydrogenase and glutamate synthetase activities of *Klebsiella*, *Azotobacter*, *Clostridium*, *Chromatium* and soybean nodule bacteroids indicated that glutamate synthetase played the more prominent role in glutamate formation.

Additional evidence favoring a correlation between glutamate synthetase and dinitrogen fixation was provided by genetic studies in which *K. pneumoniae* mutants that lacked glutamate synthetase activity were unable to grow on $N_2$, but could grow on a high level of $NH_4^+$, presumably *via* glutamic dehydrogenase. It was suggested that the absence of functional glutamate synthetase activity prevented the mutants from utilizing the low levels of ammonia which were furnished by $N_2$ reduction, and accordingly growth terminated (509).

# IV. Other Enzymes and Proteins Associated with $N_2$ Fixation

In addition to nitrogenase and its electron donors other proteins appear to be involved with the $N_2$ fixation function, though the nature of the relationship is obscure in most cases. This is true for the enzyme hydrogenase and the heme protein leghemoglobin, both long associated with $N_2$ fixation, but never in a definitive way. Similarly, variations in the cytochromes of certain diazotrophs have been correlated with growth on $N_2$ *vs.* growth on fixed nitrogen, but the significance of these differences is not obvious. Leghemoglobin and perhaps some of the cytochromes seem to have a unique relationship with $N_2$ fixation; the others are found also in cells which do not fix $N_2$, but often in relatively smaller amounts. The various proteins that have, or appear to have, ancillary functions in $N_2$ fixation are discussed below.

## A. Hydrogenase

$$2H^+ + 2e^- \rightleftharpoons H_2$$

Hydrogenase has been rigorously defined as any enzyme that catalyzes the formation and utilization of $H_2$ in a reversible manner, analogous to an $H_2$ electrode. In practice, however, the criterion of reversibility is not always respected, and the demonstration of either $H_2$ evolution or of $H_2$ uptake alone has been considered sufficient evidence for the presence of hydrogenase. Additionally, catalysis of $D_2$-$H_2O$ exchange or of *ortho-para* conversion in $H_2$ has been accepted as an adequate test for the enzyme.

Hydrogenases are found in a wide range of microorganisms where they are most commonly associated with anaerobic metabolism. They have been purified from a number of sources including the diazotrophs *Clostridium* (383, 510, 513), *Azotobacter* (330) and *Desulfovibrio* (295, 418). Non-heme iron and labile sulfide have been detected in the enzymes of *Clostridium* (510, 513) and *Desulfovibrio* (418), and Mo has been implicated in the activation of *Clostridium* hydrogenase (383). Partial characterization of the *Clostridium* enzyme shows it to have a molecular weight of 60,000 daltons, 4 Fe and 4 $S^{2-}$, an e.p.r. signal at a g-value of 1.94 in the reduced state, and an $\alpha$-helix content estimated at 11-14% (506, 510, 513).

Early studies with diazotrophs established that these organisms all showed evidence of hydrogenase activity by at least one criterion and often by several. This unusual correlation gave rise to the concept that some sort of unique relationship exists between nitrogenase and hydrogenase. However, when acellular nitrogenase preparations became available for study, it was soon established that nitrogenase itself was capable of catalyzing certain $H_2$-based reactions which had previously always been considered to be exclusively hydrogenase reactions. Specifically, nitrogenase was shown to catalyze an $H_2$ evolution reaction and a $D_2 \rightleftharpoons H_2O$ exchange reaction. The former required ATP, $Mg^{2+}$ and reductant and was called ATP-dependent $H_2$ evolution. The latter reaction required all these and additionally $N_2$ and, of course, $D_2$. It was apparent that the exchange reaction occurred only during concomitant reduction of $N_2$, and it appears highly probable that the exchange involves the hydrogen atoms of enzyme-bound intermediates of $N_2$ reduction. In addition to these hydrogenase-like reactivities, nitro-

genase exhibits another involvement with $H_2$, namely $H_2$ inhibition of $N_2$ reduction. The inhibition is competitive, indicating bonding of $H_2$ at the $N_2$ site:

$$\text{Nitrogenase}\cdot N_2 + H_2 \rightleftharpoons \text{Nitrogenase}\cdot H_2 + N_2$$

The various $H_2$ reactions associated with nitrogenase are diagrammed in Figure 8-3.

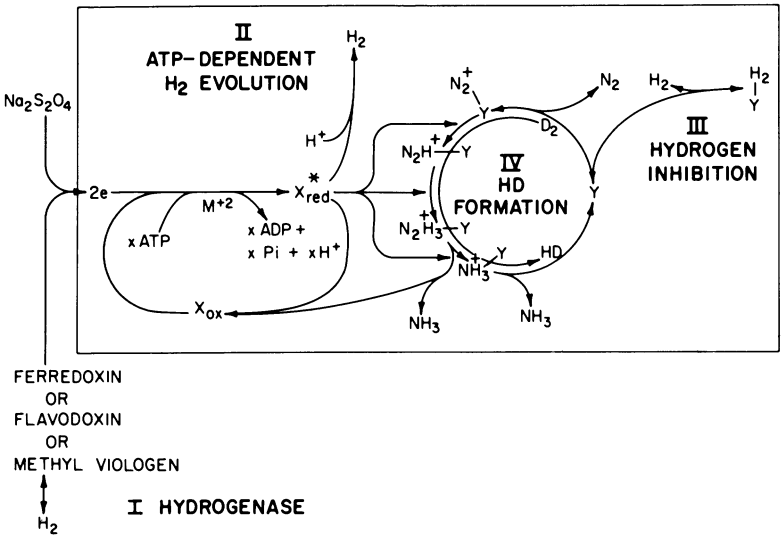



Fig. 8-3. $H_2$ involvements with nitrogenase (Ref. 339)

The ability of nitrogenase to catalyze reactions previously attributed exclusively to hydrogenase requires a re-evaluation of the evidence which led to the concept of a unique relationship between the two enzymes. In this reconsideration it is emphasized that there is no evidence yet that ATP-dependent $H_2$ evolution by nitrogenase ever occurs *in vivo*; if it does not, then clearly the $H_2$ evolution which has been observed in intact cells is attributable solely to hydrogenase, as had been initially believed. Moreover, since nitrogenase does not react with $H_2$ to produce a net uptake of $H_2$ under any known conditions, all assays based on net $H_2$ uptake may be considered to truly reflect hydrogenase activity. Finally it is clear that nitrogenase will not evolve $H_2$ in the absence of continuous supply of ATP, and thus it cannot compromise *in vitro* measurements of hydrogenase, since this nitrogenase requirement is not met under normal conditions of the hydrogenase assay, whether by $H_2$ evolution or uptake. However, it must be borne in mind that to the extent that ATP-dependent $H_2$ evolution by nitrogenase occurs *in vivo*, the use of $H_2$ evolution cannot be used as the sole criterion for hydrogenase activity in intact diazotrophs.

The concept of a hydrogenase-nitrogenase relationship dates from at least 1940 when PHELPS and WILSON (556) discovered hydrogenase activity in extracts of *Azotobacter vinelandii*, though earlier observations of $H_2$ inhibition of $N_2$ fixation may have suggested a hydrogenase involvement. PHELPS and WILSON recognized the presence of hydrogenase

in *Azotobacter* as rather remarkable, since not only is this organism a strict aerobe and thus an unlikely candidate for the enzyme, but additionally there is no evidence that it either consumes or utilizes $H_2$ in its normal metabolism. Its discoverers, no novices in the area of $N_2$ fixation, suggested the enzyme might in fact have significance for $N_2$ fixation. This concept was strengthened by the subsequent finding that *Azotobacter* cells cultured on $N_2$ possessed more hydrogenase activity than cells cultured on other nitrogen sources (417), and it became even more attractive when *Azotobacter* mutants unable to fix $N_2$ were shown to have a lowered hydrogenase content (261).

It soon became apparent that just as *Azotobacter* was an unusual possessor of hydrogenase, the hydrogenase it possessed was also rather unusual. Unlike the hydrogenases of anaerobes, which reversibly activated dihydrogen, the *Azotobacter* enzyme appeared to be strictly a "one-way" hydrogenase, capable only of activating $H_2$ for the reduction of various electron acceptors, but incapable of evolving $H_2$. On the basis of its reaction characteristics, it was suggested that the $E_o'$ of the *Azotobacter* hydrogenase is more positive than those of other organsims (400).

The unique aspects of the *Azotobacter* hydrogenase notwithstanding, the hydrogenase-nitrogenase correlations observed with this organism prompted the testing of other hydrogenase-possessing organisms for $N_2$ fixation. Though this soon established that hydrogenase was not unique to nitrogen fixers, it was an important factor leading to the successful testing of the photosynthetic bacterium, *Rhodospirillum*. The significance of establishing $N_2$ fixation with this organism already has been mentioned (p. 73). Work with diazotrophs other than *Azotobacter* has established the presence of hydrogenase activity in almost all organisms tested. An exception among the legumes is reported for lupine nodules (182), but it should be pointed out that hydrogenase activity in soybean nodules was detected only after initial negative tests (310). Among nodules of the alder type, $H_2$ could not be detected with alder, *Hippophaë* or *Casuarina* nodules (151), though a low level of $H_2$ evolution was observed with *Macrozamia* nodules (50).

The photosynthetic bacteria, exemplified by *Rhodospirillum*, show yet another side of a possible hydrogenase-nitrogenase relationship. These organisms photoevolve $H_2$ under anaerobic conditions. This activity is inhibited by $N_2$ and by $NH_4^+$, but proceeds vigorously upon exhaustion of $NH_4^+$ or when glutamate is used as the nitrogen source. These effects by $N_2$ and $NH_4^+$ suggest that nitrogenase may be contributing to $H_2$ evolution *via* ATP-dependent $H_2$ evolution; a decrease in this activity in the presence of added electron acceptors such as $N_2$ is consistent with observations of nitrogenase behavior *in vitro*. On the other hand, GRAY and GEST have suggested that $N_2$ may function as an allosteric inhibitor of hydrogenase (258). The suppressing effect of ammonia could be due to repression of nitrogenase biosynthesis. With respect to the inability of glutamate to inhibit $H_2$ evolution in *Rhodospirillum*, the observation of MUNSON and BURRIS (507) is particularly revealing. They found active nitrogenase in glutamate grown cells by $^{15}N_2$ methods, and suggested that the poor growth observed with glutamate probably reflected a difficulty in utilizing this substrate; the resulting deficiency in usable nitrogen would accordingly promote the biosynthesis of nitrogenase, and the observed $H_2$ evolution might be attributed to the activity of this enzyme. The degree to which the ATP-dependent $H_2$ evolution activity of nitrogenase might contribute to the photoevolution of $H_2$ in photosynthetic bacteria is difficult to assess from existing data, but the possibility exists that little or no hydrogenase of the *Clostridium* type is present in these organisms.

The inability of *Rhodospirillum* extracts to evolve $H_2$ from reduced methyl viologen and their ability to reduce methylene blue with $H_2$ (551) suggest that *Rhodospirillum* hydrogenase is similar to that of *Azotobacter*, but unlike that of the anaerobes. If this be the case, a nitrogenase involvement in the $H_2$ evolution of whole cells seems likely.

Studies with legume nodules have shown $H_2$ uptake by the nodules of peas and beans (182), and uptake also has been demonstrated with pea nodule bacteroids (181). The inability to show hydrogenase activity with lupine nodules was referred to above. In work with soybean nodules, $H_2$ evolution and $N_2$-dependent exchange between $D_2$ and endogenous hydrogen sources have been shown (311); $H_2$ uptake, however, has not been reported. Evolution of $H_2$ by soybean nodules is inhibited by $N_2$ and $N_2O$ (311) and the inhibition is reported to be competitive (41). The work with pea and bean nodules indicates the presence of hydrogenase in these systems; however, the reactions observed with soybean nodules are chracteristic of nitrogenase and may be due partly or completely to this enzyme rather than to a hydrogenase.

Investigations with other diazotrophs do not provide a great deal of support for a special hydrogenase-nitrogenase relationship. In the strictly anaerobic bacteria, hydrogenase plays a major role regardless of nitrogen source. *Clostridium* may in fact possess less hydrogenase when grown on $N_2$, as indicated by a comparison of extract activities (96). Intact cells of *Bacillus* and *Pseudomonas*, though, were reported to show greater hydrogenase activity when grown on $N_2$ *vs.* fixed nitrogen (571), but the possible nitrogenase contribution to $H_2$ evolution was not assessed. No whole cell comparisons appear to have been reported for *Clostridium*, but $H_2$ evolution by this bacterium is not inhibited by $N_2$ and is thus likely to be due strictly to hydrogenase. The microaerophilic diazotroph, *Mycobacterium flavum*, possesses a hydrogenase which is able to function in the utilization of $H_2$ as an electron donor for nitrogenase (58, 59). The *Mycobacterium* hydrogenase would thus appear to more closely resemble the hydrogenases of anaerobes than the hydrogenase of *Azotobacter*; however, it resembles the *Azotobacter* enzyme in its inability to evolve $H_2$ (58). Among the algal diazotrophs little work has been reported concerning even the existence of hydrogenase, much less the occurrence of a possible hydrogenase-nitrogenase relationship.

Except for one report (723) in which electron transfer from nitrogenase to hydrogenase was indicated, no functional basis has been established for a special relationship between hydrogenase and nitrogenase; however, the nature of hydrogenase suggests that it might function to protect or to regulate nitrogenase. These possibilities have little besides a certain reasonableness to support them, data being completely lacking. In view of the $O_2$ sensitivity of nitrogenase and the $O_2$-scavenging capacity of hydrogenase, it is not difficult to imagine a protective function for hydrogenase whereby $O_2$ is kept from the nitrogenase environment *via* the Knallgas reaction,

$$H_2 + 1/2\ O_2 \longrightarrow H_2O,$$

using metabolically generated $H_2$. The concept is not unlike the "respiratory protection" mechanism described by POSTGATE (p. 103). Utilization in this way of any $H_2$ formed by nitrogenase could account for the failure to detect $H_2$ evolution from *Azotobacter*. In anaerobic diazotrophs, the regulatory functions which GRAY and GEST (258) ascribe to hydrogenase could have special significance for nitrogenase. These functions are concerned primarily with energy metabolism and with the maintenance of reducing potential, both of paramount concern in nitro-

genase function. The apparent anomaly of an organism like *Clostridium* vigorously evolving $H_2$ at the same time it has a demand for reducing power to fix $N_2$ might be rationalized in terms of a regulatory mechanism which is geared to overproduce reductant (in terms of metabolic need) in order to increase ATP synthesis.

In summary the case for a unique physiological relationhsip between hydrogenase and nitrogenase appears strongest in the aerobic fixers, most specifically in *Azotobacter*. The work with *Rhodospirillum* suggests that it, and presumably other photosynthetic bacterial diazotrophs, possess a hydrogenase akin to the unusual hydrogenase of *Azotobacter*, and it also indicates that nitrogenase contributes to the $H_2$ evolved by whole cells. Studies with legume nodules do not provide a consistent picture. The $H_2$ evolution and HD exchange activity of soybean nodules suggests nitrogenase function, and true hydrogenase activity from these nodules may not yet have been detected. The $H_2$ assimilation shown for pea and bean nodules and for pea bacteroids, however, clearly reveals the presence of hydrogenase. The position of the blue-green algae is ill-defined. The non-photosynthetic anaerobic diazotrophs offer little support for a unique relationship, but an indirect relationship might be imagined in which nitrogenase is one of many enzymes subject to regulation and/or protection by hydrogenase.

## B. Leghemoglobin

Leghemoglobin is a heme protein unique to legume nodules. It appears to be specifically associated with leguminous symbiotic $N_2$ fixation, perhaps more so than any other protein except nitrogenase itself. Its localization within the nodule and the temporal and quantitative relationships between leghemoglobin and $N_2$ fixation were discussed earlier (p. 36). Though the dense red color that the protein imparts to the nodule interior must have brought it to the attention of the earliest investigators, its hemoglobin nature was not established until 1939 by KUBO (401). On occasion the protein has been reported in non-legume root nodules, but the bulk of evidence does not support this, and it seems likely that leghemoglobin is unique to legume nodules (32).

Leghemoglobin probably functions as an $O_2$ carrier specifically designed to cope with the presumed $O_2$ paradox which nitrogenase creates for the nodule interior. This arises from the dual nature of $O_2$, which acts both as a major component in energy metabolism and as an inhibitor or inactivator of nitrogenase. The high $O_2$ flux which would most effectively furnish energy for nitrogenase represents a potential threat to the sensitive enzyme, and the ability to facilitate the delivery of $O_2$ to specific sites in an environment maintained at low $pO_2$ would have obvious advantages. An $O_2$-carrying role was in fact suggested initially for leghemoglobin by KUBO and others, but firm experimental support has been difficult to obtain.

Early experiments with nodule extracts showed that leghemoglobin had a high affinity for $O_2$, becoming half-saturated at a $pO_2$ of <1.0 mmHg (365). APPLEBY (16) measured a value of 0.05 mmHg and estimated from spectral observations that intact soybean nodules contained a mixture of ferroleghemoglobin and oxyleghemoglobin leading to a low internal $O_2$ tension in the nodule. In subsequent spectral analyses APPLEBY (20, 21) concluded that *in vivo* leghemoglobin existed in the high spin $Fe^{2+}$ form characteristic of $O_2$-carrying heme proteins and was 20-25% oxygenated at ambient $pO_2$; additionally, evidence was provided that the low spin forms of leghemoglobin, which were observed earlier

and which suggested an electron carrier function for leghemoglobin (208), were artifacts of isolation (21).

Recently more extensive equillibria and kinetic analyses of the interactions of $O_2$ and CO with soybean leghemoglobin have confirmed and extended the earlier reports. Thus IMAMURA and RIGGS (331) and WITTENBERG, APPLEBY and WITTENBERG (725), have obtained well-defined rate constants and Michaelis constants as indicated in Table 8-2.

Table 8-2. Kinetic data for soybean leghemoglobin-$O_2$ binding

| | WITTENBERG, APPLEBY and WITTENBERG (725) | IMAMURA and RIGGS (331) |
|---|---|---|
| k' $O_2$ "on", $M^{-1}sec^{-1} \times 10^{-6}$ | 94-120 | 150 |
| k $O_2$ "off", $M^{-1}$ $sec^{-1}$ | 2.8-4.9 | 11 |
| $K_{O_2}$ k'/k $\times 10^{-6}$ | 20-40 | 11.6-13.6 |
| l' CO "on", $M^{-1}$ $sec^{-1} \times 10^{-6}$ | 11.6-12.8 | 13.5 |
| l CO "off", $M^{-1}$ $sec^{-1} \times 10^{-6}$ | - | 0.012 |
| $L_{CO}$, l'/l $M^{-1} \times 10^{-9}$ | - | 0.99-1.13 |
| $pO_2$, mmHg | 0.021-0.068 | 0.045-0.047 |
| pCO, mmHg | 0.001 | 0.00071-0.00074 |

Comparisons of these values with those of myoglobin or hemoglobin show that leghemoglobin has up to about 10 times greater affinity for $O_2$ and CO, and that this is due primarily to substantially greater ligand "on" constants. The authors of both papers emphasize the suitability of these leghemoglobin charactisterics for facilitating $O_2$ diffusion in the nodule. More direct evidence favoring such a role for leghemoglobin has been provided by the recent respiration studies of TJEPKEMA and YOCUM (686). Using a highly sensitive $O_2$ electrode, they measured respiration rates in nodule tissue at low $pO_2$ and showed these to be reduced by about one half when leghemoglobin was inactivated by CO.

Other roles have been proposed for leghemoglobin, but none of these has received much support. A role in $N_2$ reduction, based on the ability of leghemoglobin to complex with diazene (270), was suggested, but this proposal and all others implicating the protein directly in the $N_2$ fixation mechanism are now considered invalid since nitrogenase catalysis *in vitro* shows no requirement for leghemoglobin. An apparent $N_2$-leghemoglobin complex (1) has been ascribed to spectral complexities introduced by contaminating cytochrome (19), and this explanation may also apply to another, more recent, report of a leghemoglobin-$N_2$ complex (220). The possibility that leghemoglobin participates in electron transfer seems unlikely in view of the apparent artifactual nature of the spectral evidence which suggested cytochrome character in leghemoglobin. In addition to the apparent $O_2$-binding function of leghemoglobin it has been reported to have peroxidase activity (497, 568), but the physiological significance of this activity is not clear.

The chemical and physical nature of leghemoglobin is becoming increasingly well characterized. ELLFOLK (202) crystallized leghemoglobin from soybean nodules and showed that it consisted of two electrophoretically distinguishable components, each of which contained one Fe. Molecular weight values of 16,800 and 15,400 daltons for the faster and slower moving components, respectively, were determined (203). The two components differ in N-terminal amino acids with glycine in the faster and valine in the slower, but they have similar amino acid compositions, and neither contained cysteine nor methionine (204, 205). The amino acid sequence of the slower moving component has been determined (209). The hemin of each component was identified as protoheme by spectral measurements and titration data (206). Analyses of artificial leghemoglobins composed of various hemins plus the apoprotein emphasized the importance of propionate groups in forming a stable complex (207).

Nodules of lupine and *Seradella* each contain two major leghemoglobin pigments (172). These have been partially characterized (174), and lupine leghemoglobin has been crystallized (533).

Most quantitative estimates of leghemoglobin are based on pyridine hemochromogen procedures such as that described by VIRTANEN et al. (696). These have been criticized by WILSON and REISENAUER (712), who described an alternate colorimetric method in which leghemoglobin is measured in the cyanmetleghemoglobin form. A modification of this method has been used to evaluate the $N_2$-fixing ability of peanut nodules (596).

The biosynthesis of leghemoglobin is of particular interest. Like the nitrogenase proteins, it is synthesized only by the functioning symbiosis. Evidence from several sources is consistent with a biosynthetic pattern in which the genetic information for apoprotein synthesis is carried by the plant, while heme synthesis occurs in the bacteroids, though the latter point seems less well established (248). This concept is supported by the specific localization in bacteroids of the heme-synthesizing enzymes (338) and of the activity for incorporation of $^{14}$C-δ-amino levulinic acid into leghemoglobin (140). In contrast, material which cross-reacted with antibodies prepared from highly purified leghemoglobin components was uniquely found in the nodule cell cytoplasm (139). The question of genetic control was examined in an elegant experiment by DILWORTH (172) in which a) two different legumes species were inoculated by a single *Rhizobium* strain and b) samples of a single legume species were inoculated with either of two different strains of *Rhizobium*; subsequent comparisons of the chromatographic elution patterns of the purified leghemoglobins produced by these combinations showed that the leghemoglobin elution pattern was characteristic of the legume and independent of the *Rhizobium* component. Results obtained subsequently with snake bean (83) and other legumes (141) support the earlier work. These experiments demonstrate that genetic control of apoleghemoglobin resides in the plant genome, though the site of synthesis may occur in either the plant cell or the bacteroid.

## C. Other Proteins

### *1. Cytochromes*

APPLEBY (17, 18) compared the cytochrome complements of *Rhizobia* growing freely in culture and *Rhizobium* bacteroids isolated from soybean

nodules. Perhaps the most striking difference was the apparent absence in bacteroids of cytochrome oxidases, which were readily identified in the cultured cells; however, the bacteroids uniquely contained auto-oxidizable pigments identified as cytochrome c (552, *Rhizobium*) and hemeprotein P-450. Additionally, pigments P-420 and P-428 were found exclusively in the bacteroids. A hemeprotein designated "*Rhizobium* hemoglobin" was observed uniquely in the cultured cells, but appears to be unrelated to leghemoglobin. At present the significance of these observations to $N_2$ fixation, or their relevancy to bacterial *vs.* bacteroid metabolism, remains obscure.

Several investigators have observed a higher level of cytochromes in *A. vinelandii* grown on $N_2$ *vs.* cells grown on fixed nitrogen (see for example, Refs. 390, 391, 675). These differences do not appear to arise from any obvious relationship between cytochromes and nitrogenase, but could reflect higher energy needs of cells growing on $N_2$ (675). A cytochrome effect of a different type was observed in the protective influence exerted by cytochromes against cold or storage inactivation of purified nitrogenase (370; also p. 104). The requirement for cytochrome was non-specific, mammalian cytochrome c being as effective as *Azotobacter*. YATES has reported that the $C_2H_2$-reducing activity of *A. chroococcum* extracts is stimulated by diverse proteins including cytochrome $c_4$, NADH dehydrogenase and an iron-sulfur protein (736). Additionally NADH dehydrogenase protected nitrogenase against $O_2$ inactivation. The significance of these protein effects on nitrogenase remains difficult to assess.

*2. Nitrate Reductase*

An apparent anomaly in the enzyme composition of legume nodules, as reported by CHENIAE and EVANS (129). The nitrate reductase activity of soybean nodules was positively correlated with various parameters of $N_2$ fixation, such as leghemoglobin content. The role of this enzyme is unknown, and its presence in nodules remains a curious phenomenon, since there is no evidence suggesting conversion of ammonia to nitrate in nodules.

The co-occurrence of nitrate reductase and nitrogenase in nodules has led to the suggestion that there is a Mo-containing component common to both enzymes (217). Though some experimental support for such a component has been obtained with preparations from diverse sources (515), no evidence of its existence in nodules has been presented.

*

In conclusion, our understanding of the biochemistry and biophysics of the biological $N_2$-fixing reaction has come a long way in recent years. Section II of this volume has attempted to assemble this information. It is obvious that many aspects of $N_2$ fixation are now unequivocally defined; it is equally obvious that many more problems have been revealed and much still remains to be done.

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